DIFFERENTIATION AND PROPERTIES OF AMNIOTIC FLUID CELLS

by

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Thesis presented for the degree of Doctor of Philosophy

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June, 1982

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Abbreviations

AF	-	amniotic fluid cell type
AFC	-	amniotic fluid cells
BCG	-	bacillus Calmette-Guèrin
BLP	-	blue latex particles
CNS	-	central nervous system
CSF	-	cerebro-spinal-fluid
DNA	-	deoxyribonucleic acid
E	-	epithelioid cell type
EARFC	-	sensitized erythrocyte rosette-forming cells
F	-	fibroblastoid cell type
FCS	-	fetal calf serum
FD	-	fetal distress
GFAP	-	glial fibrillary acidic protein
hCG	-	human chorionic gonadotrophin
HEPES	-	N^1-2-Hydroxyethylpiperazine-N^1-Ethanesulphonic acid
LETS	-	large external transformation sensitive
NSAE	-	non-specific acid esterase
NTD	-	neural tube defect(s)
PAS	-	periodic acid-Schiff
PBS	-	phosphate buffered saline
RBC	-	red blood cells
RYLP	-	red-yellow latex particles
UV	-	ultraviolet

ACKNOWLEDGEMENTS

I am particularly indebted to: my supervisors Drs. D.J.H. Brock and C.M. Gosden for their guidance and encouragement throughout this project; to Dr. J.B.L. Bard for his assistance with cine-micrographic techniques and all his helpful suggestions; and to Dr. W.H. McBride for performing the Fc receptor assay, for all his support and valuable suggestions.

I am most grateful to Lilias Barron, Patricia Eason, Caroline Hayward, Sandra Lourie and Suzanne Thompson for their help and patience in the performance of this work; to Norman Davidson and Sandy Bruce for their excellent advice and technical assistance on photography and art work; to Sheila Mould and Helen Moffat for all their help in obtaining the bibliography; to T. Johnston for making amniotic fluid cultures available; to Dr. J. Bell for providing some fetal material; to Drs. Gordon and Maloney for helpful discussion; to Professor M. Ferguson-Smith for providing some amniotic fluids; to Dr. S. van Heyningen for his gift of tetanus toxin and equine antiserum and to Mrs. Norquay for typing this manuscript.

I am indebted to Professor H.J. Evans for all his support throughout this project.

Finally I would like to thank my family and friends for their support and encouragement, especially my fellow Ph. D. students; Steve Campbell and David Brook. Steve, for introducing me to the study of cell biology, for all his help and suggestions and both Steve and David for sharing three years of "scientific research"

with the successful as well as disappointing moments.

It would be an impossible task to thank every single person who has been at any time of help during the performance of this work, so I would like to apologise for all the omissions and acknowledge everyone in the MRC Unit and Human Genetics Department for making the past three years as instructive and enjoyable as possible.

During the performance of this study I have been supported by a scholarship from the Mexican National Council for Science and Technology.

To the memory of my grandfather

Serafin Gómez Galindo

(1905-1981)

I hereby declare that this thesis has been prepared by me and is the product of my individual effort. The work included in this thesis has not been presented for any other degree or professional qualification elsewhere.

ABSTRACT

Amniotic fluid cells have been widely used, initially in the assessment of gestational age and Rhesus isoimmunization; more recently in the antenatal diagnosis of chromosomal disorders, inborn errors of metabolism, haemoglobinopathies, some congenital malformations and detection of high risk pregnancy. However, the amniotic fluid cell origin is not yet clear.

This study examined the properties of uncultured and cultured amniotic fluid cells, as well as their differentiation through culture. Amniotic fluids were divided into normal and abnormal according to their alpha fetoprotein values (Brock, 1981) and the following cell characteristics were examined: numbers, morphology, viability, adherence, phagocytosis, presence of Fc receptors, non-specific acid esterase, behaviour in vitro by cinemicrography and immunocytochemistry.

The heterogeneity of amniotic fluid cells was confirmed and their origin is discussed. The use of the previously neglected uncultured amniotic fluid cells is enhanced. The presence of macrophages in normal (Hoyes, 1968) and abnormal (Sutherland et al., 1973, 1975) amniotic fluids was confirmed. Macrophages were increased in number in amniotic fluids from fetuses with anencephaly but not in those with spina bifida. The macrophages were short-lived and incapable of survival and proliferation in culture. The presence of neural cells in amniotic fluids from fetuses with a neural tube defect (Gosden and Brock, 1978a) was also confirmed. The neural cells consisted of neuroblasts which differentiated through culture into neurons; glial cells which seem to dedifferentiate

into glioblasts; and microglia.

The studies in normal amniotic fluids suggested that the main cell type (AF) of long-term amniotic fluid cell cultures originates from trophoblast (Priest et al., 1979) and that their heterogeneity is due to differentiation in culture from cytotrophoblast into syncytiotrophoblast.

The implications of these findings in antenatal diagnosis are discussed.

I. INTRODUCTION

A. Use of Amniotic Fluid Cells in Antenatal Diagnosis

Amniotic fluid cells (AFC) are fetal in origin (Huisjes, 1978). Much of the interest in AFC derives from their use in antenatal diagnosis of fetal abnormality. AFC are widely used in diagnosis of cytogenetic disorders (Bowser-Riley, 1978), inborn errors of metabolism (Patrick, 1978) and recently in some haemoglobinopathies (Alter, 1981). Cell culture of AFC, which is complicated and expensive, is usually required in order to obtain enough cells for their analysis. Rapidly-adherent AFC, cultured for 20 hours, have recently been used as an aid in the antenatal diagnosis of congenital malformations (Gosden and Brock, 1978a) and in monitoring high risk pregnancies (Gosden and Brock, 1978b). The use of uncultured AFC has instead been neglected.

The following research project will investigate the properties of either uncultured or cultured AFC in an attempt to improve their use in antenatal diagnosis.

B. Uncultured Amniotic Fluid Cells

Amniotic fluid cytology was first studied in the early years of this century, but it was not until a half century had elapsed that clinical applications were found. Most of the first studies were

done in uncultured cells of third trimester pregnancies as an aid in the diagnosis of ruptured membranes, severe Rhesus isoimmunization and determination of gestational age.

The contribution of epithelial cells to the amniotic fluid in the second and third trimesters of pregnancy has been well studied (Huisjes, 1970, 1973, 1978; Cohen, 1971; Casadei et al., 1973; Morris and Bennett, 1974; Cutz and Conen, 1978). Most investigators have classified the cells as derived from fetal epidermis, amnion, and the mucosa of digestive and respiratory tracts and urogenital epithelia. However, more recent studies using scanning electron microscopy have found that at mid-trimester there are no cells in amniotic fluid from amniotic membranes (Bergström, 1979; Tyden et al., 1981) or from the respiratory tract and urinary bladder (Tyden et al., 1981). Instead, the last three authors found detachment of cellular fragments from periderm and umbilical cord during a limited period in mid pregnancy, and exfoliation of whole cells from squamous epithelia like vagina, nasal mucosa, oral mucosa down to the epiglottal tip and skin during peridermal regression. The different origin of the cells in amniotic fluid can explain in part their heterogeneity, but other factors influencing the cellular content of amniotic fluid are the time of gestation (Hoyes, 1968; Huisjes, 1978) and sex of the fetus (Tyden et al., 1981).

Uncultured AFC have been surprisingly neglected in antenatal diagnosis during the second trimester of pregnancy. Their study requires no specialized or expensive culture techniques and only a small amount of amniotic fluid is necessary, regardless of cell

viability. The examination of sex chromatin in uncultured AFC is sometimes used as a secondary test in the antenatal diagnosis of fetal sex, but the karyotype of cultured AFC is considered more reliable and is always performed.

Regarding fetal pathology, Pasquinucci et al. (1969) in their study of uncultured amniotic fluid samples between 21 and 42 weeks of gestation found that 17 out of 90 showed the presence in the sediment of unclassified cells and abundant cellular debris. Of these 17 cases, 15 were from patients carrying fetuses with severely affected Rhesus isoimmunization. They suggested that this could indicate a stage of "fetal suffering" and that further research associating cytologic and clinical findings was needed.

Recently, the study of uncultured AFC has been suggested as an aid in the antenatal diagnosis of neural tube defects (NTD) in the fetus. Huisjes (1973) made the first observations of a giant multinucleated cell found in an uncultured amniotic fluid from a fetus with anencephaly. Papp and Bell (1979) studied smears of uncultured AFC from normal fetuses and fetuses with a NTD. The uncultured AFC from normal fetuses consisted mainly of squamous cells of varying sizes, some histiocyte-like cells, some macrophage-like cells and few elongated slender cells. In contrast, AFC from fetuses with anencephaly contained more macrophage-like and elongated slender cells. In 5 cases where the fetus had spina bifida, only one had increased numbers of macrophage-like cells and two had a slightly higher proportion of elongated cells. Haemosiderin and lipid material were demonstrated in some of the

macrophages.

Another similar study was performed by Chapman et al. (1981) in cytospin preparations of uncultured AFC, who classified the cells as squamous cells, amnion cells, macrophages, large macrophages and neural cells. Amniotic fluids from normal fetuses contained mainly squamous cells, some amnion cells, and some macrophages, while only 1.5% of the amniotic fluids had large macrophages. Their cases of amniotic fluids from fetuses with either anencephaly or anencephaly and spina bifida contained not only the same cell types as amniotic fluids from normal fetuses but also had a large number of large macrophages and neural cells. The macrophages often contained lipofuscin and/or finely vacuolated lipid, while glycogen was sometimes present and in a few cases haemosiderin was found. Large foamy macrophages were found containing large amounts of lipid.

1. Macrophages in uncultured amniotic fluids

Macrophages in uncultured AFC from normal fetuses have been described. Hoyes (1968), in his study of the ultrastructure of uncultured cells of amniotic fluids from 16 to 32 weeks of gestation, refers to a type of cell that varied with the gestational age. In specimens from younger fetuses, they were relatively small with a rounded and eccentric nucleus, well-developed Golgi apparatus and a number of membrane-bound bodies containing material of varying electron density in the cytoplasm. In samples from older fetuses, the cells were larger and of a more variable form, while a normal Golgi apparatus was rarely present and the cytoplasm contained numerous membrane-bound bodies and an increased amount of cellular

debris. Examination in the electron microscope revealed that these cells were capable of phagocytic activity and resembled macrophages of the lung and peritoneal cavity. Their origin was not clear, but Hoyes thought they were derived from amniotic epithelium. They were rarely found after the 32nd week of pregnancy. He explains this with three theories: 1) the amniotic epithelium differentiates and is incapable of releasing macrophages; 2) the cells are disrupted; and 3) the cells are swallowed by the fetus.

Casadei et al. (1973), in their study of uncultured amniotic fluid cells from 16 to 22 weeks of gestation, also refer to the occasional finding of macrophages with a spongy vacuolated material and phagocytic properties. These could be single or multi-nucleated with finely vacuolated cytoplasm and eccentric oval or bean-shaped nuclei.

Recently, Cutz and Conen (1978), in their study of uncultured amniotic fluids between 14 to 16 weeks of gestation, selected only fluids free of blood contamination and found three different types of cells classified according to their characteristics by light, transmission and scanning microscope. Epithelial cells were characterized as keratinizing or non-keratinizing and comprised 80 to 95% of the non-viable cells in amniotic fluid as shown by trypan blue staining. In contrast, the viable cells were mainly of two other types. One type had morphologic and functional characteristics of macrophages and were shown to phagocytose in vitro both latex particles and bacteria. They showed a strong acid phosphatase reaction and numerous phagosomes. Although apparently normal constituents of amniotic fluid, their origin was not clear. These cells were easily found in cell samples up to 20 weeks gestation, but

were less numerous during later gestations. The other type of cell appeared singly or in clusters and resembled the lining of the amnion. They contained cytoplasmic glycogen and fine regular surface microvilli, but lacked phagosomes and phagocytic capacity *in vitro*.

Cutz and Conen suggest the interesting possibility that amniotic fluid macrophages could contribute to the defence system of the fetus as the immune system and the inflammatory response are not fully developed in the early stages of gestation.

The neural lesion in fetuses with a NTD has been suggested by both Papp and Bell (1979) and Chapman *et al.* (1981) as the possible origin of the macrophages and neural cells present in their amniotic fluids. Papp and Bell (1979) also suggest amnion as another possible origin of macrophages.

2. Neural cells in uncultured amniotic fluids

The neural cells present in uncultured amniotic fluids from fetuses with a NTD have been described as elongated slender cells (Papp and Bell, 1979) or as individual and clustered small cells with dark nuclei and little or no cytoplasm (Chapman *et al.*, 1981). Chapman *et al.* (1981) found no evidence for differentiation of these cells either by electron microscopy or by staining for glial fibrillary acidic protein (GFAP), a specific marker for glial cells, astrocytes (Antanitus *et al.*, 1975), ependymal cells (Roessmann *et al.*, 1980) and cells of the enteric nervous system (Jessen and Mirsky, 1980). In contrast, Sarkar *et al.* (1980), in their

immunocytochemical study of uncultured AFC from a fetus with anencephaly, found no staining for protein 14-3-2 (neuronal-specific enolase), but positive staining for protein S-100 which is present in glial cells.

C. Cultured Amniotic Fluid Cells

The cells of amniotic fluid are difficult to culture, for although the number of viable cells present at the time of amniocentesis is 15-36%, the plating efficiency is very low (Nelson, 1973; Hoehn et al., 1974; Cutz and Conen, 1978; Martin, 1980). In spite of the wide use of second trimester amniotic fluid cell cultures in antenatal diagnosis, the lack of knowledge with regard to the characteristics and behaviour of different cell types in culture has led to various classifications (Table 1).

1. Rapidly-adherent amniotic fluid cells

Amniotic fluid cells that adhere rapidly to glass (20 - 24 hours) have proved to be of value in antenatal diagnosis of some congenital malformations and in the identification of pregnancies where there was a risk of eclampsia, abortion or low birthweight infants (Gosden and Brock, 1978a,b). Many of these rapidly-adhering cells found in short term cultures have vacuoles and inclusions and, when initially described, were classified as macrophages (Sutherland et al., 1973, 1975). Instead, recent reports have classified the cells by morphology into epithelioid,

TABLE 1 AMNIOTIC FLUID CELLS IN CULTURE: CLASSIFICATION

Authors	Cell types observed	
	20-24 h	Long-term cultures > 1 week
Gerbie et al., 1972 Nadler, 1972 Hasholt, 1976		Epithelial-like Fibroblast-like
Sutherland et al., 1973 1975	Macrophages	Epithelioid Type I II III Fibroblast-like
Hoehn et al., 1974 Megaw et al., 1977 Priest et al., 1977a,b 1978, 1979, 1980 Crouch et al., 1978		Epithelioid-like (E) Fibroblast-like (F) Amniotic fluid (AF)
Gosden and Brock, 1978a,b	Rapidly-adherent cells: Epithelioid Placental Neural Peritoneal Fetal distress	
Virtanen et al., 1981b		Epithelial - E1, E2, E3, E4, E-5 Fibroblasts

neural, peritoneal, placental and fetal distress cells (Gosden and Brock, 1978a,b).

a) Morphological classification of rapidly-adherent AFC

i) Epithelioid cells: These are large pale-staining squamous cells which usually have a small nucleus. The classification as epithelioid by Gosden and Brock (1978a), although correct because most of these cells desquamate from several epithelia (section I, B), is confusing due to the fact that the term epithelioid is widely accepted in long-term cultures (including amniotic fluids) for cells with entirely different characteristics. Throughout the present study, these large pale-staining squamous cells will be referred to as squamous cells.

ii) Neural cells: These comprise long cells of both bipolar and fibroblastic types, cells with fine filamentous pseudopodia, large vacuolated cells and multinucleated giant vacuolated cells. These cells have been studied in more detail by gold/palladium cell shadowing for surfaces using transmission and scanning electron microscopy (Gosden et al., 1979).

iii) Placental cells: These differ from neural cells in lacking cytoplasmic granulations and inclusions. The most characteristic feature is an eccentrically placed nucleus appearing as a cap at one end of the cell. Occasionally multinucleates are found, but these are small 'groups' which lack vacuolations and cytoplasmic bridges.

iv) Peritoneal cells: There are two main groups: cells with eccentrically placed nuclei, and peritoneal macrophages which have prominent nucleoli and lace-like cytoplasm without any inclusions.

Binucleated cells are common and large cells with several nuclei are occasionally seen.

v) Fetal distress cells type I: They are large, round cells with a quite prominent cell membrane. The nucleus is invariably small, intensely stained and the central cytoplasm is diffuse with no prominent features.

vi) Fetal distress cells type II: They are small, especially when compared with neural cells. Their shape may vary considerably although many are triangular or square. The nucleus is central or near to the centre and usually contains prominent nucleoli. The most characteristic feature is large irregular vacuoles in the cytoplasm, usually placed immediately adjacent to the nuclear membrane.

Gosden and Brock (1978a,b) used cell adherence and cell morphology as an aid in the antenatal diagnosis of fetal congenital abnormality, high risk pregnancy and high risk newborn (Table 2). Subsequent studies of rapidly-adherent amniotic fluid cells (Medina-Gómez, 1979) proved that some of these cells were highly phagocytic.

b) Phagocytic properties of rapidly-adherent AFC

i) Phagocytic cells: These form the first line of host defence against toxic and foreign materials. The cells participating in this process are very heterogeneous but form two main systems: the polymorphonuclear leukocytes of the blood (neutrophils, eosinophils and basophils) and the mononuclear phagocyte system (macrophage and precursor cells) distributed in the blood and fixed in tissues (van Furth et al., 1972; Gordon and Cohn, 1973).

TABLE 2 RAPIDLY-ADHERENT CELLS IN AMNIOTIC FLUIDS

Pregnancy Outcome	Cell Adherence (20-24 h)	Cell Types
Normal singletons > 2500 g	< 6%	Squamous Occasional placental Occasional fibroblasts
Spontaneous abortion, low birthweight and severe pre-eclampsia		Fetal distress Occasional placental
NTD:		
Anencephaly	30-100%	(Neural
Spina bifida	9-25%	(Occasional placental
Omphalocele	10%	Peritoneal
NTD and Omphalocele	53%	Neural and Peritoneal

Polymorphonuclear cells are wandering cells that remain in the blood from 9 to 26 hours and then migrate from the vessel walls into the tissue, i.e. skin and mucous membranes. The monocytes, in contrast, reside in the circulation for 2 to 3 days and then migrate to tissues where they become tissue macrophages, long-lived cells that can endure for months under steady-state conditions. Injury to tissues causes an inflammatory response, with first polymorphonuclear leukocytes migrating from the blood to the source of irritation, and then macrophages entering the area (Weir, 1977).

Macrophages are fairly large cells (10-25 μm) with single reniform or oval nuclei and prominent nucleoli. The cytoplasm has granules and vacuoles and the plasma mebrane shows extensive ruffling and many microvilli. Their functional characteristics are firm adherence to glass or plastic surfaces and avid pynocytosis and phagocytosis. The term activated macrophages is applied to macrophages from recently infected animals which phagocytose and destroy the infecting microorganism more rapidly and efficiently than do macrophages from uninfected animals. Activated macrophages have an enhanced bactericidal activity; adhere more strongly to glass or plastic surfaces; increased capacity for phagocytosis; exhibit more intense undulatory movements of the cell membrane and have an increased number of hydrolytic enzymes, metabolic enzymes and adenylcyclase (North, 1978; Mota, 1980).

Macrophages are an important link between innate and acquired immunity. They regulate interaction with antigen in part by selecting the key antigenic determinants. Macrophages have an

antigen presenting function which is a sophisticated and elaborate process. This function is essential in promoting growth of selective lymphoid cells that recognize antigen in the context of proper molecules on the macrophage. The phenotypic expression of these molecules is controlled by genes of the major histocompatibility complex. Macrophages modulate immune induction as regulator cells by the extent to which lymphocytes are stimulated, and as effector cells by responding to signals from stimulated lymphocytes (Nathan et al., 1980; Unanue, 1980, 1981).

ii) Phagocytosis: The process of phagocytosis can be separated into four stages: (van Furth and van Zwet, 1973)

1) *attachment of the particulate matter to the cell surface*
2) *engulfment of the particle:* the phagocytic cell surrounds the particle by emitting pseudopods which eventually meet and fuse forming the phagocytic vacuole or phagosome, with the wall being the inverted membrane
3) *intracellular killing of microorganisms*
4) *digestion of microorganisms and other ingested matter*

These last two stages include the contact of the phagosome with adjacent lysosomes, the fusion of its membranes, followed by rupture of the common membrane and discharge of lysosomal enzymes into the phagosomes.

Phagocytosis is facilitated by opsonins, i.e. serum components such as immunoglobulins (specific antibodies), complement and other less well-known serum factors that mediate the recognition of the foreign particles by the phagocytic cells (van Furth and van Zwet,

1973; Mota, 1980).

Temperature also seems to be important. It has been shown that in the kinetics of phagocytosis of Staphylococcus aureus and E. coli in human granulocytes, at temperatures of 4 to 33^{0}C, there is a diminished function of the cells that leads to a diminished phagocytosis. Above 42^{0}C the opsonization is affected and it has only a slight influence in the ingestion process. The maximum ingestion was observed between 33 and 39^{0}C (Leijh et al., 1979).

Information about the phagocytic process has been obtained by in vivo or in vitro techniques, the first being based on the measurement of the clearance of substances injected into a living animal. This method has the disadvantage of being influenced by many unknown variables such as humoral factors, blood flow, concentration of particles, and number and functional state of the phagocytic cells. In vitro studies have the advantage of known homogeneous populations with well-defined and characterized phagocytes and particles, while the effects of serum concentration, temperature and other factors can be controlled (van Furth and van Zwet, 1973; Leijh et al., 1979).

Phagocytosis has been studied in vitro by cells attaching to glass or plastic surfaces (Michell et al., 1969) or in suspensions (Conen and Cutz, 1978). The number of particles, microorganisms, yeast cells, latex particles or oil droplets ingested per cell can be determined microscopically and their phagocytic indices calculated (Capo et al., 1974).

iii) Phagocytic properties of rapidly-adherent AFC and their measurement: Rapidly-adherent AFC have been tested for phagocytosis (Medina-Gómez, 1979). The cells were classified according to Gosden and Brock (1978a,b) except for fetal distress (FD) cells type II which were further subdivided into three groups, while only cells with branching processes were labelled as neural cells (Fig. 1).

Three different types of latex particles were used, the difference between them being their colour and size: blue latex particles (average diameter 5.7 μm), yellow latex particles (average diameter 0.806 μm) and red latex particles (average diameter 0.234 μm). When scoring was carried out under the light microscope, it was observed that the ingested latex particles lost their colour. Red and yellow latex particles were not identifiable by size so they were grouped together and scored as Red-Yellow latex particles.

The degree of phagocytosis was measured as +, ++ and +++. Blue latex particles (BLP) were counted easily and, with a maximum of 8 in a cell, were scored as follows: 1 - 2 (+), 3 - 4 (++), 5 or more (+++). Red-Yellow latex particles (RYLP) were more difficult to count and the scoring was more subjective. Up to about 100 particles was labelled as +, double the amount as ++, and +++ for a cell full of latex particles where the number was uncountable.

It was observed that the phagocytic cells could be divided into three groups according to the type of latex particles ingested. In Table 3, the phagocytic index and degree of phagocytosis are given separately for BLP only, for RYLP only and for all latex particles. The phagocytic index was calculated by dividing the

FIG 1: Phagocytic properties of rapidly-adherent amniotic fluid cells.

Non-phagocytic cells:

a) Squamous cell
b) Fetal distress cell type I

Phagocytic cells:

c) Fetal distress cell type II-a
d) Fetal distress cell type II-b
e) Fetal distress cell type II-c
f) Neural cell type

(from Medina-Gómez, 1979)

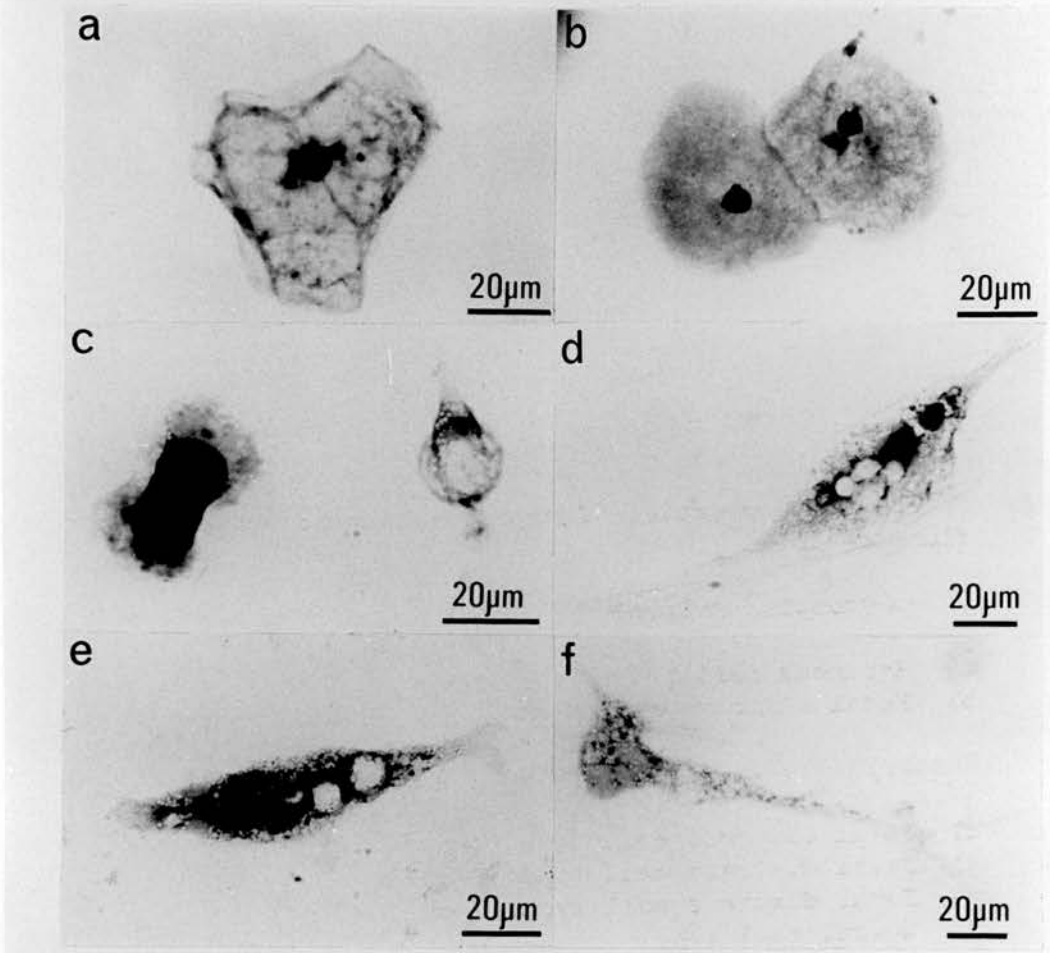

a
20μm
b
20μm
c
20μm
d
20μm
e
20μm
f
20μm

TABLE 3 PHAGOCYTIC INDICES AND DEGREE OF PHAGOCYTOSIS IN AMNIOTIC FLUID CELLS

Type of cell	Blue Latex Particles		Red-Yellow Latex Particles		All Latex Particles		Total Phag. Index	
	Phag. Index	Deg. of Phag.	Phag. Index	Deg. of Phag.	Phag. Index	Deg. of Phag.		
Squamous	0%		0%		0%		(476)	0%
Placental	0%		0%		0%		(1)	0%
FD I	0%		0%		0%		(14)	0%
FD II a)	(15) 3%	+	(223) 52%	++	(98) 23%	++/1	(429)	78%
b)	(93) 13%	+++	(441) 61%	+++	(143) 20%	++/3	(721)	94%
c)	0%		(27) 63%	++	(16) 37%	++/1	(43)	100%
Neural-like	0%		(10) 71%	++	(4) 29%	+/1	(14)	100%

Phagocytic Index = $\frac{\text{Phagocytic cells}}{\text{Total cells}}$

Degree of Phagocytosis - Blue Latex Particles (BLP)
1-2 (+)
3-4 (++)
5 or more (+++)

- Red-Yellow Latex Particles (RYLP)
$\leq$ 100 (+)
100-200 (++)
> 200 (+++)

- All Latex Particles
RYLP/BLP

cells which phagocytosed latex particles by the total number of cells with or without phagocytic properties. In this table the number of cells in which the phagocytic index was calculated is in brackets.

Cells which were classified as FD II had the highest phagocytic activity. These cells were present in amniotic fluids from normal fetuses and in two amniotic fluids from fetuses with a NTD, one with anencephaly and the second with an atypical encephalocele.

c) Immunocytochemistry of rapidly-adherent AFC

The origin of rapidly-adherent cells in amniotic fluids from normal fetuses and fetuses with a NTD has been recently tested by immunocytochemical methods.

i) Immunocytochemistry: This is the study by immunologic methods of the cellular structure observed by light and/or electron microscopy. The specificity of this analysis depends on the primary binding of antibodies; the visualization of this specific binding can be enhanced by several methods which have been extensively reviewed by Sternberger (1974).

One of these methods consists of making antibody fluorescent by conjugating it with a fluorescent group. Immunofluorescence can be further subdivided into six methods according to the staining sequence for localization of the antigen-antibody reaction.

1) direct technique: tissue antigen binds fluorescein labelled specific antibody.

2) indirect technique: this is the most widely used technique in immunofluorescence. In the first step, tissue antigen binds

specific unlabelled antibody from species "a". In the second step, the bound unlabelled antibody binds the fluorescein labelled second antibody to the immunoglobulin of species "a" produced in species "b".

3) *complement technique:* in the first step, tissue antigen binds specific antibody (IgG or IgM). In the second step, complement is applied in the form of guinea pig serum in the presence of calcium and magnesium. In the third step, fluorescein conjugated immunoglobulin against guinea pig complement is bound.

4) *staining of intracellular antibody:* detection of intracellular antibody simply by addition of fluorescein labelled specific antigen is not a very sensitive method.

5) *double staining technique:* antigen "a" stains green by binding antibody from fluorescein labelled anti "a"; antigen "b" in the same tissue stains orange red by binding antibody from lissamine rhodamine B 200 labelled anti "b".

6) *sequential technique:* in the first step, antigen "a" binds fluorescein labelled "a", the staining pattern is photographed and the fluorescence is destroyed by prolonged exposure to the full beam of the ultraviolet light. In the second step, a second antigen "b" is localized with fluorescein labelled anti "b" and photographed.

ii) Immunocytochemistry of rapidly-adherent AFC: Immunocytochemistry has been used to detect the presence of intermediate filaments and fibronectin.

Intermediate filaments are present in mammalian cells and have a diameter of 100 Å. They have been named intermediate filaments because their diameter is larger than small actin filaments (60 Å) and smaller than larger myosin filaments (150 Å) or microtubules (250 Å) (Lazarides, 1980). Intermediate filaments are a distinct fibrous system of chemically heterogeneous sub-units which divide into five major classes: 1) keratin (tono) filaments found in epithelial cells and cells of epithelial origin; 2) desmin filaments, found predominantly in smooth, skeletal and cardiac muscle cells; 3) vimentin filaments, found in mesenchymal cells and cells of mesenchymal origin; 4) neurofilaments, found in neurons and 5) glial filaments, found in all types of glial cells (Lazarides, 1980; Virtanen et al., 1981a (Table 4).

Fibronectin or LETS protein (Large External Transformation Sensitive) is a large external surface protein, fibrous in nature, that seems to be a structural component of the embryonic extracellular matrix. Like collagen, it is distributed in different amounts in various tissues and plays a role in the development as well as the function of the organ in question (Hay, 1980). Although fibronectin is mainly expressed in fibroblast or fibroblast-like cells (Schachner et al., 1978), it is also present or produced by some epithelial cells (Chen et al., 1977), endothelial cells (Bidwell et al., 1978), macrophages (Nathan et al., 1980), meningeal cells in culture (Fields, 1979) and E, F and AF types of amniotic fluid cells in long-term cultures (Crouch et al., 1978). In fact, Crouch et al. (1978) found a higher release of fibronectin into the culture medium of AF cell types (48%) and E cell types

TABLE 4 PRESENCE OF INTERMEDIATE FILAMENTS IN DIFFERENT CELL TYPES

Cell type	Vimentin (MW 52,000)	Desmin (MW 50,000)	Keratins (MW 40,000-65,000)	Glial filaments (GFA) (MW 51,000)	Neurofilaments (MW 200,000 150,000, 68,000)
Muscle:					
Skeletal, cardiac, smooth	+	+	-	-	-
Myotubes	+	+	-	-	-
Myoblasts	+	+	-	-	-
Mesenchymal and ectodermal cells:					
Human fibroblasts	+	-	-	-	-
Avian fibroblasts	+	+*	-	-	-
3T3	+	+	-	-	-
SV40-3T3	+	ND	-	-	-
BHK-21	+	+	-	-	-
NIL-8	+	-	-	-	-
Sarcoma 180	+	ND	-	-	-
Rhabdomyosarcoma	+	+	-	-	-
Schwann cells	+	+	-	-	-
Neuroblastoma C1300	+	-	-	-	-
Pheochromocytoma PC12	+	-	-	-	+
Pigment cells	+	-	-	-	-
Skin melanocytes	+	ND	-	-	-
Chondroblasts	+	-	-	-	-
Endothelial cells	+	-	-	-	-
PNS, CNS neurones	-	-	-	-	+
Glial cells:					
Astrocytes) Oligodendrocytes) Microglia) Ependymal cells) Glioma C6)	+	-	-	+	-
Epithelial cells:					
Amnion epithelial cells:					
Primary cultures	+*	-	+	-	-
Subcultures	+	-	+	-	-
Hela	+	-	+	-	-
Rat kangaroo PtK_1, PtK_2	+	-	+	-	-
Epidermal cells	-	-	+	-	-
Cultured keratinocytes	+	-	+	-	-
Stratified squamous epithelia	-	-	+	-	-
Epidermal appendages	-	-	+	-	-
Hassal's corpuscles of the thymus	-	-	+	-	-
All other epithelial cells	+	-	+	-	-
Exceptions:					
Epithelia of iris and lens	ND	-	-	-	-
Glomerular and tubular cells of kidney	ND	-	-	-	-
Liver hepatocytes	+	ND	+	-	-
Hepatoma cells	+	ND	-	-	-
Pancreas	ND	ND	-	-	-

* Some cells ND - not determined.

From Lazarides, 1980 and Virtanen et al., 1981

(41%) than F cell types (19%).

The results of immunocytochemical studies on the origin of rapidly-adherent amniotic fluid cells are summarized in Table 5.

2. Long-term cultures

The report by Steele and Breg (1966) of the successful culture and karyotype of cells in amniotic fluids has led to the widespread use of cultured cells in antenatal diagnosis of cytogenetic disorders (Bowser-Riley, 1978). Recently, antenatal diagnosis has been performed in inherited disorders where there is a chromosome marker such as fragile X chromosome in X-linked mental retardation (Jenkins et al., 1981) or an increased number of chromosome breaks in Fanconi anaemia (Voss et al., 1981). Antenatal diagnosis is also feasible in inborn errors of metabolism (Brock, 1978; Patrick, 1978; Galjaard, 1980), some haemoglobinopathies (Alter, 1981) and in some diseases with HLA linkage such as congenital adrenal hyperplasia (Pollack et al., 1979) and complement C 4 deficiency (Pollack et al., 1980).

AFC for antenatal diagnosis can be cultured in one of two general methods: 1) in situ on coverslips; 2) mass culture, usually in flasks and requires trypsinization to obtain a cell suspension.

The first method is considered more reliable for chromosome analysis and "potentially error free" (Hecht et al., 1981), because it differentiates true chromosome mosaics from pseudomosaics that arise by tissue culture. The average length of time in culture

TABLE 5 ORIGIN OF RAPIDLY-ADHERENT AMNIOTIC FLUID CELLS BY IMMUNOCYTOCHEMISTRY

Author	Fetal diagnosis	Gestation (weeks)	Type of culture	Time in culture	Fibronectin	Keratin	Vimentin	GFAP	Neuro-filament
Aula et al., 1980	Anencephaly	23	P	24 h		+		+ (only a proportion)	
	Normal	?	P	24 h (?)					
Cremer et al., 1981a	Anencephaly	18	P	4-6 days *)	Few % after 4 days, increase with time in culture			+ (4%)	
	Anencephaly	25	P	18 days *) +				+ (1 sparse colony)	
	Encephalocele	30	P	4-6 days *)				+ (80%)	
	Omphalocele	16	?	?				-	
	Normal	17	P	several ** weeks	+ (consid-erable proportion)			-	
von Koskull et al., 1981	Anencephaly	19	P & S	P: 24 h			+	+ (80% or more and	
	Anencephaly	17	P	48 h		- (24 h)	+	+ (dominating cell (type up to 7	
	Anencephaly	16	P	every 3 days		+ (1% at 7	+	+ (weeks	+
	Normal	15-16	P	up to 28 days		days or more)	+	-	

** Medium was first changed after 5 days instead of 24 h as in NTD cases (*).

P - primary culture

S - subculture

required to obtain sufficient cells for a karyotype is 14 to 21 days (Bowser-Riley, 1978) and up to 8 weeks for enzymatic assays in inborn errors of metabolism (Patrick, 1978). Recently developed microtechniques have diminished the time in culture to 1 to 2 weeks for some inborn errors of metabolism (Galjaard, 1980).

The importance of the cell type that is being analysed was first stressed by Gerbie et al. (1972), who found different cytologic and biochemical characteristics of epithelioid-like and fibroblast-like cells. Other influencing factors in the assay of enzymes for antenatal diagnosis have been described by Littlefield (1971) and Galjaard (1980): contamination by maternal cells or fungi and bacteria; cell heterogeneity; effects of the culture media; stage of cell cycle at harvesting; cell density; time in culture and the period between seeding and analysis; and the enzyme assay per se. With the recent use of microtechniques, early cultures of AFC have shown large variations which are not related to time (Henkels-Dully and Niermeijer, 1976). In various cell types, microchemical assays and interferometric measurements of the cellular dry weight have proved that lysosomal enzyme activities are the same when expressed per unit of dry weight but there are large differences when the activity is expressed per cell (van der Veer et al., 1978). All these reasons contribute to the importance of having proper amniotic fluid controls for antenatal diagnosis.

The most used classification of AFC in long-term cultures was first described by Hoehn et al. (1974). He introduced a new cell type, the amniotic fluid (AF) cell. Table 6 lists the main

TABLE 6 CHARACTERISTICS OF AMNIOTIC FLUID CELLS IN LONG-TERM CULTURES

Epithelioid - E	Amniotic Fluid - AF	Fibroblast Type - F	Reference
24%	66.5%	8.8%	
Cellular pleomorphism < AF Multinucleation > AF Intimate cell to cell contact Sheet-like coherence	Cellular pleomorphism Tendency to multinucleate Loose network at confluence	Cells of spindle shape No tendency to multinucleate Parallel arrays at confluency	Hoehn et al., 1974
Lowest longevity	Growth potential less than half of human diploid monolayer fibroblast-like culture	Growth potential of other human diploid monolayer fibroblast-like cultures	
Resistant to trypsin detachment	Subcultured by trypsin	Subcultured by trypsin	
Complete morphological concordance between E-type colonies derived from fetal urines and those from amniotic fluids or kidney dissociation	AF colonies from fetal urine did not fully express the characteristic "bull's eye" pattern of vigorous mature AF amniotic fluid colonies.		Hoehn et al., 1975
Kidney specific LDH not present	Kidney specific LDH not present		
	Production of epithelial basement membrane glycoprotein	No production of epithelial basement membrane glycoprotein	Megaw et al., 1977
	Biochemical evidence for basement membrane collagen	Biochemical evidence for type I collagen	Priest et al., 1977a, b 1978 Priest & Priest, 1980
	Extracellular material with ultrastructural characteristics of basement membrane collagen	Extracellular material with ultrastructural characteristics of type I fibroblast collagen	
	Differentiated hormone production (Human Chorionic Gonadotrophin) e.g. Progesterone	No production of Human Chorionic Gonadotrophin	Laundon et al., 1981
	Original tissue source could be trophoblast	Original tissue source is fibroblastic	

TABLE 6 (contd.)

Epithelioid - E	Amniotic Fluid - AF	Fibroblast Type - F	Reference
Fibronectin synthesized and released to culture medium 41%	Fibronectin synthesized and released to culture medium 48%	Fibronectin synthesized and released to culture medium 19%	Crouch et al., 1978
	Type IV like procollagen (AF_1) Procollagen with three identical pro alfa chains structurally and immunologically related to the pro alfa 1 chains of type I procollagen (Pro AF_2).	Predominant synthesis of type I procollagen. Smaller amounts of type III procollagen.	Crouch & Bornstein (1978, 1979)
	Derived from trophoblast, show cyto and syncytio trophoblast morphology.	Previous "stromal" cells of placental cultures.	Priest et al., 1979
	Binucleates arise by fusion		Priest et al., 1980

characteristics of AFC in long-term cultures. However, Virtanen et al. (1981b) have reclassified AFC using antibodies against intermediate filaments in indirect immunofluorescence microscopy (Table 7).

TABLE 7 CLASSIFICATION ACCORDING TO THE PRESENCE OF INTERMEDIATE FILAMENTS IN NORMAL AMNIOTIC FLUIDS (normal karyotype and AFP)

Hoehn et al., 1974		Virtanen et al., 1981b					
		Size & Morphology	Presence in AFC cultures	Intermediate Filaments			
				keratin	vimentin	desmin	GFAP
AF	E-1	middle size, pleomorphic	In all AF cultures as major cell type	+	+	-	-
E	E-2	large, flat	Low numbers in all cultures	+	+	-	-
	E-3	middle size, pleomorphic	In isolated clones in a small proportion of cultures	+ (desmosomes)	+	-	-
	E-4	small, dense colonies		+ (desmosomes)		-	-
	E-5	large, multinucleated cells	Occasionally in cultures	+	+	-	-
F	Fibroblasts	fibroblastoid or middle size, pleomorphic	Exceptionally as major cell type	-	+	-	-

II AIMS OF THE STUDY

When this research project was started, information on AFC concerned either uncultured cells, or cells adhering at 20 hours, or long-term cultures. The main aim of this study was to link these separate observations by studying the properties of AFC from normal and abnormal fetuses both when uncultured and through varying periods of time in culture.

Phagocytic experiments with rapidly-adherent AFC (Medina-Gómez, 1979) suggested that Sutherland et al (1973, 1975) had correctly classified these cells as macrophages. However, morphological studies by Gosden et al (1977) and initial cinemicrographic results of AFC from fetuses with a NTD (Medina-Gómez, 1979) also suggested that there were neural cells present, even though the number of cells with neural characteristics was very variable.

In order to identify and quantify amniotic fluid macrophages, specific properties of macrophages were investigated. These included minimum time of adherence and phagocytosis, the presence of non-specific acid esterase and the presence of Fc receptors. The identification of neural cells in amniotic fluids was attempted by use of cinemicrography and immunocytochemistry. Previous studies on AFC have used mainly fixed cells, so cinemicrography had an important role in giving information on the behaviour of live cells in culture.

The aims of the characterization and dynamic studies of AFC were to improve antenatal diagnosis techniques, to cast some light on the aetiology of congenital malformations and the pathology of

pregnancy, and by increasing information on the development of the fetus and the pregnancy, to improve the chances of the safe delivery of a normal healthy baby.

III MATERIALS AND METHODS

A. Materials

1. Amniotic fluids

Amniotic fluids were obtained between 16 and 22 weeks of gestation, during amniocentesis or prior to intra-amniotic infusion of prostaglandins for termination of pregnancy. The amniotic fluids have been classified as normal and abnormal according to AFP values (Brock, 1981). In the abnormal amniotic fluids, the fetal defect was confirmed after termination of pregnancy. In some of those with normal AFP values, the pregnancies are still in progress.

A total of 104 amniotic fluids have been studied. The 49 abnormal amniotic fluids have been divided into four main groups according to the main fetal abnormality: 1) NTD: a) anencephaly or anencephaly and spina bifida; b) spina bifida; c) encephalocele; 2) omphalocele or gastroschisis; 3) NTD and omphalocele or gastroschisis; 4) other congenital malformations.

Omphalocele is a condition in which there is herniation of the abdominal contents through a midline defect; the protruding viscera are covered by a membrane. Gastroschisis has a paramedium localization without a membranous sac. However, differential diagnosis of gastroschisis is difficult and not recognised by all specialists as an independent clinical entity (Colombani and Cunningham, 1977) so in this study both entities will be grouped as

omphalocele.

The experimental studies were always limited by the small amounts of amniotic fluid available. The routine diagnosis (antenatal diagnosis of NTD and fetal karyotype) had priority and only amniotic fluids obtained prior to termination of pregnancy could be tested in several ways (Table 8).

2. Fetal cells

Fetal lung fibroblasts were grown in culture from explants of fetal lung from fetuses spontaneously aborted.

Fetal neural cells were obtained from fetal brain of fetuses aborted for social reasons. Other neural fetal cells such as encephalocele cells, spinal cord and cerebro-spinal-fluid (CSF) were obtained from fetuses aborted as a result of antenatal diagnosis of fetal malformation (Table 9).

B. Methods

1. Cell counts and viability

Gosden and Brock (1978a) found no difference in total amniotic fluid cell numbers between cells from normal and abnormal fetuses. In contrast, Sarkar et al. (1980) found differences in cell number and DNA content between normal amniotic fluids and one amniotic fluid from a fetus with anencephaly. Using a Coulter counter for

TABLE 8 STUDIES OF NORMAL AND ABNORMAL AMNIOTIC FLUIDS

No.	Gestation (weeks)	AFP Kiu/ml	Diagnosis	Adherence + Phagocytosis	Fc Receptors	NSAE	Morphology in LTC	Cine-micrography	Immuno-cytochem. Tet. Toxin
I. NTD									
A. Anencephaly (A)									
A-1	18	NT	A					X	
A-2	20	78	A	X				X	
A-3	19-20	59.5	A	X				X	
A-4	18	392	A + Cervical S.B.			X		X	X
A-5	16	NT	A + R	X	X			X	X
A-6	16	266	A	X					
A-7	20+	NT	A	X		X			
A-8	20	NT	A		X				
A-9	19	150	A		X	X			
A-10	18	143	A			X	X		
A-11	18	NT	A + R			X			
A-12	17	222	A			X			
A-13	16	175	A			X	X		
A-14	20	124	A				X		
A-15	21	199.6	A + Cervical S.B.			X			
A-16	18	235.8	A + R			X			
A-17	17	241.5	A						
A-18	16	218	A			X		X	
A-19	18 (S)	218.4	A			X		X	
A-20	18	101.4	A			X	X	X	
A-21	19	206.5	A + R				X		

(S) - Gestational age given by scan
NT - Not tested
SB - Spina bifida
R - Rachischisis
I - Iniencephaly
LTC - Long-term cultures

TABLE 8 (Contd.)

No.	Gestation (weeks)	AFP Kiu/ml	Diagnosis	Adherence + Phagocytosis	Fc Receptors	NSAE	Morphology in LTC	Cine-micrography	Immuno-cytochem. Tet. Toxin
B. Spina Bifida (S.B.)									
S-1	18(S)	21.39	Lumbar S.B.					X	
S-2	21	49.5	I + thoraco lumbar S.B.						X
S-3	29	97.5	I + S.B.			X	X		
S-4	18	36	Lumbosacral S.B.			X			
S-5	18	100	Thoraco lumbar S.B.			X		X	
S-6	18	55	Thoraco-lumbo-sacral S.B.				X		
S-7	17	63.5	Double S.B.)thoracic)lumbar			X	X	X	
S-8	16(S)	56.2	Thoraco-lumbo-sacral S.B.			X	X		
S-9	18(S)	44.7	Thoraco-lumbar S.B.			X			
S-10	18(S)	68.4	Lumbo-sacral S.B.			X	X	X	
S-11	18-19	70.2	Thoraco-lumbo-sacral S.B.					X	
S-12	22(S)	20.4	Thoraco-lumbar S.B.			X			
C. Encephalocele (E)									
E-1	19	NT	E		X				
E-2	25	47	Meckel Syndrome		X		X		
E-3	18(S)	98.5	Meckel Syndrome		X				
E-4	17	268	E & R		X				
E-5	20(S)	18.9	Skin covered E attached to placenta. Severe midline facial cleft & cleft palate. Amniotic adhesion with reduction deformities of left hand		X				
E-6	20	79.2	E		X				

TABLE 8 (Contd.)

No.	Gestation (weeks)	AFP Kiu/ml	Diagnosis	Adherence + Phagocytosis	Fc Recepiors	NSAE	Morphology in LTC	Cine-micrography	Immuno-cytochem. Tet. Toxin
II. Omphalocele and Gastroschisis (O)									
O-1	18	186	Gastroschisis				X		
O-2	21	154	Gastroschisis	X		X	X	X	
O-3	16	378	Severe omphalocele Multiple congenital abnormalities including amputation of right arm and other digits and syndactyly.			X			
O-4	23	68.3	Omphalocele			X			
O-5	17(S)	281.6	Omphalocele				X		
III. NTD and Omphalocele (NO)									
NO-1	20	308	A + R + O			X		X	
NO-2	Amniocentesis - 20	197.2	I + E + cyclopia + O			X			
	Termination - 22 Intact Sac	202				X		X	
NO-3	18	144.6	A + O				X		
NO-4	19	265	A + R + O				X		
IV. Other Abnormalities (Ot.)									
Ot.-1	Amniocentesis - 19	30.7	No NTD No structural defects			X			
	Termination - 20	N.T.	Extensive skin damage			X			
V. Normal Amniotic Fluids (C)									
C1-54	16-22)	within	Delivery of normal infants or pregnancy						
	)	normal	still in progress		(10)	(29)	(30)	(8)	
C-55	19)	limits	Cystic Fibrosis			X	X	X	

TABLE 9 STUDIES PERFORMED ON FETAL CELLS

Cell type	Gestation (weeks)	Diagnosis	Morphology in culture	NSAE	Cine-micro-graphy	Immunocyto-chemistry Tetanus toxin
Fetal lung fibroblasts	12-18	Normal fetuses	(5)	(1)		(4)
Fetal brain	12	Normal fetus	X	X		X
	14	Normal fetus	X			
	15	Normal fetus	X			
Encephalocele	25	Meckel syndrome E-2	X	X	X	
	22	Cyclopia + Encephalocele + Omphalocele NO-2	X	X	X	
Spinal cord	16	Anencephaly A-13		X		
Cerebro-spinal fluid	18	Spina bifida with Arnold Chiari S-6		X		

( ) - No. of cases studied

determining relative cell volume, Sarkar et al. (1980) found an unusual peak of cells in the amniotic fluid from the fetus with anencephaly which was in slight excess of 50% of the total number of cells in that sample. Using flow microfluorometry, they also found a wide range of relative DNA content in the amniotic fluid from the fetus with anencephaly due to the presence of multinucleated cells.

Rapidly-adherent AFC, as used by Gosden and Brock (1978a,b), select for viable cells that adhere and remain attached to glass for 20-24 hours of culture. The following experiment was designed to find out if there was a significant difference in cell viability of AFC from 19 normal fetuses and 8 from abnormal fetuses.

Cells were classified as squamous and non-squamous except for amniotic fluid A-12, where only a small aliquot was available. In this case, the routine cell counts, where no distinction between squamous and non-squamous cells is made, were considered. Non-squamous cells comprised rounded cells of varying sizes.

Cell viability and cell counts were scored in unfixed cells using a haemocytometer following the method described by Steele and Breg (1966). In order to compare cell counts using another method, cytospin preparations of AFC were studied.

2. Cytospin preparations

Cells were attached to glass slides by use of a cyto-centrifuge and 0.25 ml of amniotic fluid or fetal cell suspension was spun down at 700 rpm for 5 min. In cases of severe red blood cell (RBC) contamination, several dilutions of amniotic fluid (up to

1 in 10) were made. The slides were allowed to dry and then fixed for 10-15 min, one with cold methanol for Giemsa stain and the other with cold acetone for detection of non-specific acid esterase (NSAE) stain. Cell counts were made on cells stained either with Giemsa or NSAE.

a) Giemsa stain

Squamous and non-squamous cells in cytospin preparations stained with Giemsa were counted in one field near the centre of the cytospin preparation in order to compare them with the haemocytometer cell counts.

b) Non-specific acid esterase (NSAE) stain

All the non-squamous cells were considered in the counts.

3. Cell culture

a) Amniotic fluids

i) Rapidly-adherent AFC: Cells were plated in tissue culture of varying sizes according to the amount of amniotic fluid available and using glass coverslips as substrate: for 8-well tissue culture plates, 22 mm square coverslips and for 24-well tissue culture plates, 12 mm round coverslips were used. Amniotic fluid cells were plated with F 10 medium buffered with HEPES containing 20% fetal calf serum (FCS) in a dilution of 1:10. No matter how long the cells were kept in culture, the first medium change was done at 20-24 h after the culture had been initiated.

ii) Short-term cultures: AFC were plated as described above and kept in culture for varying periods of time up to 6 days, but half the amount of initial medium was removed and fresh medium added around the third day of culture.

iii) Long-term cultures: AFC were cultured in tissue culture flasks or dishes containing coverslips. Medium supplemented with 25% FCS was used following the method described by Bowser-Riley (1978). Half the medium was removed and fresh medium added at 5-7 days, and total medium changed every 3 days thereafter. Only amniotic fluid 0-1 had been subcultured.

b) Fetal cells

Fetal cells were obtained from fetuses aborted spontaneously or for social reasons. Fresh material is rarely available, and the prostaglandins used in inducing abortion cause the tissues to deteriorate, yielding a very low percentage of viable cells. In a few cases where the fetus had been received within a maximum of 24 h of abortion, and where the fetal suspension contained at least 50% of viable cells, cultures were initiated. The best result was obtained with a fetus that had been aborted by hysterectomy.

Fetal cell suspensions were made mechanically by suspending fetal brain or spinal cord in culture medium and then passing through a 19 gauge needle several times. The suspension was plated and cultured in the same way as amniotic fluids.

4. Adherence and phagocytosis

Previous experiments proved that rapidly-adherent AFC have

phagocytic properties (Medina-Gómez, 1979). The rapid adherence to glass and the ability to phagocytose are recognised properties of macrophages (Gordon and Cohn, 1973).

Experiments were designed to find out the minimum time for adherence and phagocytic properties of AFC. Amniotic fluids from fetuses with anencephaly have more adhering cells than normal fluids (Gosden and Brock, 1978a), and three of these were studied. For each amniotic fluid, two wells were plated in 24-well tissue culture plates as described previously. One well was used as a control and the other to test for phagocytosis. The medium of these two wells was transferred to the next two wells using new sterile plastic Pasteur pipettes after every 10 min of incubation over a period of 2 h. Fresh medium was added to the previous wells. After the initial 2 h, all the wells were incubated for 20 to 22 h and 0.1 ml of a suspension of latex particles (section I, C.1) was added to the experimental wells.

After the addition of latex particles, all the wells were incubated for a further 20 h, making a total of 40-44 h of incubation. Two other wells were set up as controls, where the medium was not transferred. They were incubated along with the other coverslips for 20-24 h, and then latex particles were added to the experimental well and both incubated for a further 20 h. All the coverslips were rinsed simultaneously with normal saline solution, fixed with formalin and stained with Leishman stain.

5. Fc receptors

Macrophages, monocytes and promonocytes have been included in a single system, the mononuclear phagocyte system. Their characteristics are avid phagocytosis and pinocytosis and the ability to attach firmly to glass (van Furth et al., 1972; Gordon and Cohn, 1973). Monocytes and macrophages have specific receptor (Fc receptor) sites for immunoglobulins and complement at the cell surface, which classify these cells as "professional phagocytes" in distinction from "facultative" phagocytic cells such as fibroblasts, reticular cells and endothelial cells which can ingest particles at a low rate (van Furth et al., 1972). Mature macrophages in tissue culture express Fc receptors that serve as a useful marker of cell specificity. Fc receptors are found on all macrophages and are stable during continued cultivation and under a wide variety of conditions (van Furth et al., 1972; Rabinovitch and De Stefano, 1973). The structural aspects and heterogeneity of immunoglobulin Fc receptors have been recently reviewed by Unkeless et al. (1981).

Fc receptors were measured following the method described by Moore and McBride (1980). Amniotic fluid obtained prior to termination of two fetuses with anencephaly was studied. The assay required 5×10^4 viable cells per well which implied 5 ml of amniotic fluid in A-5 and 4 ml in.A-8. After the amniotic fluid was spun down, 5×10^4 AFC were resuspended in 2.5 ml of F 10 medium buffered with HEPES and supplemented with 20% FCS, and then plated in a 24-well tissue culture plate. The cells were in culture for 24-48 h before the Fc receptors were measured. Since routine amniocentesis yields approximately 15-20 ml of amniotic fluid, and the cells are

used for karyotyping, it was not possible to perform this assay in control amniotic fluids. It is also known that in normal amniotic fluids cells do not attach to the substrate before 3 to 5 days in culture (Bowser-Riley, 1978; Cremer et al., 1981). For these reasons the measurement of Fc receptors was attempted in only 11 amniotic fluids using total amniotic fluid with the AFC in suspension.

6. Non-specific acid esterase (NSAE) stain

Non-specific esterases appear to be lysosomal enzymes. About 80% of monocytes have a strong or moderately strong esterase stain and about 20% show weak granular reactions (Hayhoe and Quaglino, 1980). Kaplow (1975) suggested that changes in esterase activity might reflect physiological or immunological stimulation of monocytes, probably toward their maturation to macrophages.

Non-monocytic normal haemic cells with strong positivity for non-specific esterase are megakaryocytes and platelets. Other positive patterns are present in "null" cells which show some fine positive granules, and in T cells with a strong localized "dot" positivity. Positive haemic cells present in pathological conditions are megaloblasts, red cell precursors in Di Gugliemo's disease, plasma cells of myelomas, and monoblasts and hairy cells in leukaemias (Hayhoe and Quaglino, 1980). Non-specific esterase is also said to be present in a significant number of trophoblastic cells (Wood et al., 1978).

Fifty amniotic fluids were studied. According to the amount of amniotic fluid available, AFC were tested uncultured by use of

cytospin preparations, or in culture or both. Uncultured and cultured fetal neural cells were also studied. Cytospin preparations were fixed with cold acetone and cultured cells were fixed with cold methanol, dried and stored for varying periods of time. NSAE activity was detected following the method of Mueller et al. (1975), but with staining for 3 h only. As positive controls, smears of peripheral adult and fetal blood were used while, as negative controls, cultured fetal lung fibroblasts were used. Only non-squamous cells were considered in the counts. Cells that were stained intensely red or had large strongly positive granules were counted as positive, while pale pink or green cells were counted as negative.

7. Immunofluorescence for detection of tetanus toxin receptors

Indirect immunofluorescence has been used to prove that tetanus toxin is a specific histologic marker for neurons (Fields et al., 1978; Mirsky et al., 1978; Raff et al., 1979). Tetanus toxin is a highly potent neurotoxin produced by the Gram positive bacterium Clostridium tetani (Wendon, 1979). At the time that this study was undertaken there were no available data on the use of tetanus toxin in human fetal neural tissue. Previous studies had been performed in chick, mouse and rat tissues. Thus, indirect immunofluorescence for tetanus toxin was performed in cultures of dorsal root ganglia of chick embryo, human fetal brain, fetal lung fibroblasts and three amniotic fluids from fetuses with a NTD. Rapidly-adherent AFC were tested in two amniotic fluids at 20 h and

3 or 4 days in culture. In addition, in one amniotic fluid, untreated and polylysine coated glass coverslips were used as substrate.

Polylysine coated coverslips have been used recently by Sensenbrenner et al. (1978), Petman et al. (1979) and Yavin and Yavin (1980) to grow nervous cells of rat fetuses and chick embryos. The polylysine substrate causes a rapid attachment of neurons and has a further advantage of giving a minimal presence of non-neuronal cells by inhibiting the growth of glial cells. Petman et al. (1979) used tetanus toxin as a histological marker for neurons to identify the cells growing on the polylysine substrate. Following these observations, amniotic fluid was plated in poly-L-lysine-coated coverslips using poly-L-lysine of molecular weight 80,000 (Sigma) and following the technique of Petman et al. (1979). After 3 days in culture, indirect immunofluorescence was performed.

Indirect immunofluorescence was used following the method described by Mirsky et al. (1978) and Fields et al. (1978). Purified tetanus toxin and equine antiserum against tetanus toxin (gift of Dr. S. van Heyningen) were used at a concentration of 10 µg/ml and a dilution of 1:50 respectively. The fluorescein labelled rabbit anti-horse IgG (Miles) was used at a dilution of 1:20.

8. Cinemicrography

Cinemicrography has been used to study the behaviour of live cells in culture. AFC were studied as rapidly-adherent cells, in short-term cultures of up to 6 days and in long-term cultures ranging from 6 days to several weeks.

The films were made using Wild time-lapse equipment and a Wild M 40 inverted microscope fitted with x 6 and x 10 phase-contrast objectives, the equipment being kept in a 37^{0}C hot room. The culture dish was humidified by enclosing it in a plastic case surrounded by humidified sponge. Lapse rates ranged from 10 seconds (in the study of phagocytosis) to 4 min (to examine very slow movement), the usual lapse rate being between 30 and 100 seconds per frame, while in general a higher magnification was used for a shorter lapse rate. Efforts were made to film the region of the culture where there were many adherent cells. A summary of the cultures and the extent of filming is given in Tables 19, 20, 21, 22, 24, 26, 27 and 28.

a) Rapidly-adherent and short-term AFC cultures

Thirteen amniotic fluids were studied, 4 from normal fetuses and 9 from abnormal fetuses. The cells were cultured on glass coverslips using one of the following methods:

i) <u>When more than 1 ml of amniotic fluid was available</u>: A coverslip (23 mm x 23 mm, No. 1), previously washed in alcohol, was polished with sterile gauze and sterilized in hot air, and then placed over a drop of sterile saline solution in a 6 cm Cooper dish and immobilized with Gurisil 575 G (Flexible Silicone-Rubber, Gurit-Essex). Amniotic fluid (1-2 ml) was spun down and resuspended in 0.5 ml of HAM's F 10 medium buffered with HEPES and supplemented with 20% FCS. The suspension was placed on the coverslip and the Cooper dish lid pressed down to form a large drop on the coverslip. The dish was then incubated in a humid atmosphere

at 37^{0}C for 1-2 h, after which the lid was removed and 5 ml of the same 20% FCS medium added carefully around the coverslip (so as to cause minimum disturbance to the cell suspension on the coverslip) and the lid replaced. Finally, the culture was placed on the phase-contrast microscope for cinemicrography, allowed to settle for 20-30 min and a field chosen for filming.

ii) When less than 1 ml of amniotic fluid was available: The second method for culturing the amniotic fluid was that of Gosden and Brock (1978a,b), who plated the amniotic fluid cells diluted 1:10 with 20% FCS medium directly into a glass coverslip. This coverslip was removed for filming, fixed to a Cooper dish with Gurisil, and 5 ml of fresh 20% FCS medium added. The dish was then placed on the phase-contrast microscope for cinemicrography as in method i.

For studies of phagocytosis, the medium was changed and 0.06 ml of a suspension of blue latex particles were added to the dish (~1.2 x 10^5 particles, average diameter of 5.7 μm).

b) Long-term cultures

i) Amniotic fluid cultures: Fourteen amniotic fluids were studied, 9 from fetuses with a NTD and 5 from fetuses with amniotic fluid AFP levels within normal limits (Brock, 1981). Amniotic fluid samples were obtained by amniocentesis or prior to intra amniotic infusion of prostaglandins for termination of pregnancy. The one exception was the case of the fetus with cyclopia; this was aborted as an intact sac from which the amniotic fluid was obtained. Amniotic fluids for karyotyping were cultured in Petri dishes containing

coverslips using the method described by Bowser-Riley (1978). Once the karyotype had been obtained ($\geq$ 2 weeks), the remaining coverslips were transferred to a Cooper dish as described earlier.

ii) Cultured cells from fetal encephalocele: Two of the fetuses which had an encephalocele were studied. The first had cyclopia, omphalocele and encephalocele (NO-2), while the second had Meckel syndrome (encephalocele, polydactyly and polycystic kidneys (E-2)). An incision was made in the encephalocele and fluid and cells were allowed to flow to a sterile tube. The cells were centrifuged and resuspended in 20% FCS medium, and then cultured and studied in the same way as amniotic fluids.

IV RESULTS

A. Uncultured Amniotic Fluid Cells

1. Morphology

Uncultured AFC from normal and abnormal amniotic fluids were studied by two methods: the haemocytometer and cytospin preparations. Both methods showed cells with the same morphology: squamous cells and rounded (non-squamous) cells which varied in size. The second method had the advantage of giving permanent preparations.

Normal amniotic fluids contained mainly squamous cells and cytospin preparations stained with Giemsa showed the presence of very few non-squamous cells (Fig. 2).

Abnormal amniotic fluids contained more non-squamous cells than normal amniotic fluids. Amniotic fluids from fetuses with omphalocele contained varying amounts of amorphous material (Fig. 2) which on examination in the transmission electron microscope showed fibres with the periodicity of collagen (Gosden and Ross, unpublished results). Amniotic fluids from fetuses with a NTD contained the cell types described by Chapman et al. (1981) (Fig. 2). Clusters of non-squamous cells were observed mainly in amniotic fluids from fetuses with anencephaly. The main difference between the haemocytometer and cytospin preparations was that clusters of non-squamous cells of 2 to 20 in number were observed in the haemotycometer preparations, but up to several hundreds in the cytospins. The cell

clusters had both viable and non-viable cells.

2. Viability

The results on cell viability in 19 normal amniotic fluids, 5 amniotic fluids from fetuses with a NTD and 2 from fetuses with omphalocele are summarized in Table 10. The results were analyzed statistically using the Mann-Whitney U non-parametric test (Siegel, 1956) because of the high variation in values.

Squamous cells had a lower percentage of viable cells than non-squamous cells, with a maximum of 35% in a normal amniotic fluid. This result confirms previous findings by Cutz and Conen (1978). There is a significant difference in viability between squamous and non-squamous cells in normal amniotic fluids (< 0.002) and in amniotic fluids from fetuses with a NTD (0.016). Only 2 amniotic fluids from fetuses with omphalocele were tested, and although the number is too low to allow testing of statistical difference, the mean value of viability of non-squamous cells (74%) is much higher than the value for squamous cells (20.5%). This suggests that non-squamous cells will give rise to the few cell colonies present in early cultures of amniotic fluids.

3. Cell counts

Results of the cell counts using the two methods, haemocytometer and cytospin preparations, were analyzed separately and are summarized in Table 11. Using the haemocytometer no significant difference was observed either between squamous and non-squamous cells

TABLE 10 CELL VIABILITY

	Squamous Cell Viability(%)		Non-squamous Cell Viability(%)		Total Cell Viability(%)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Controls (19)	11.53	8.38	58	25.46	28.79	21.95
NTD (5)	20.8	8.96	61.6	23.53	43.6	12.12
Omphalocele (2)	20.5	13.44	74	36.77	31	11.31

Squamous cell viability = $\frac{\text{Viable squamous cells}}{\text{Total squamous cells}}$

Non-squamous cell viability = $\frac{\text{Viable non-squamous cells}}{\text{Total non-squamous cells}}$

Total cell viability = $\frac{\text{Total viable cells}}{\text{Total cells}}$

( ) - No. of cases studied

TABLE 11 CELL NUMBERS

	Cytospin (cells/field)		Haemocytometer ($x10^3$/ml)	
	Mean	S.D.	Mean	S.D.
Normal Amniotic Fluids (19)				
Squamous cells	175	175	73	52
Non-squamous cells	5	6	33	32
Total cell count	180	176	106	66
N.T.D. (5)				
Squamous cells	213*	220	36	20
Non-squamous cells	293***	339	132	237
Total cell count	506**	291	168	234
Omphalocele (2)				
Squamous cells	323	250	55	9
Non-squamous cells	80***	37	21	14
Total cell count	403	212	76	23

* p = .05 ** p =< .02 *** p =< .002 (for a Two-tailed Test using the Mann-Whitney Test)

or between the different groups of normal and abnormal amniotic fluids. In contrast, measurements on cytospin preparations showed a significant difference between the number of squamous and non-squamous cells, but only in normal amniotic fluids (0.002). Cytospin preparations also showed a significant increase in the number of squamous cells in amniotic fluids from fetuses with a NTD and of non-squamous cells both in amniotic fluids from fetuses with a NTD and fetuses with omphalocele in comparison to normal amniotic fluids.

The high number of non-squamous cells in both amniotic fluids from fetuses with a NTD and fetuses with omphalocele suggests that the origin of most of the non-squamous cells is the fetal lesion. However, neural or peritoneal cells may not slough off as easily as epithelioid squamous cells from the surface of the lesion.

Cell counts performed in the haemocytometer agree with previous results obtained by Gosden and Brock (1978a) using the same method. However, the present study indicates that haemocytometer counts are deficient. Small non-squamous cells are not easily distinguished from RBC and big clusters of non-squamous cells, which are not seen in the haemocytometer, are frequently observed in cytospin preparations. This suggests that there is a limit to the sensitivity of the haemocytometer concerning cell or cell cluster size.

These results would explain the apparent disagreement on total cell counts between normal amniotic fluids and amniotic fluids from fetuses with a NTD obtained by Gosden and Brock (1978a) using the haemocytometer, by Sarkar et al. (1980) using a Coulter counter and

by Chapman et al. (1981) using cytospin preparations. The conclusion is that cytospin preparations will give a better image of the cell content in amniotic fluids with the further advantage of giving a permanent preparation.

4. Fc receptors

Ten normal amniotic fluids and one from a fetus with anencephaly (A-9) were studied. Fc receptor avidity was measured as described by Moore and McBride (1980) scoring the percentage of sensitized erythrocyte rosette-forming cells (EARFC). The errors in measuring EARFC were large because of the small number of non-squamous cells and the large amount of debris. The average percentage of EARFC was 60% and the mean EARFC was 9.1×10^3 per ml $\pm$ 7.52 (95% confidence limits). The amniotic fluid from the fetus with anencephaly (A-9) was "normal" to "low".

5. Non-specific acid esterase (NSAE) stain

Squamous cells were negative for NSAE stain (Fig. 2). All the non-squamous cells on the cytospin slides were scored as positive or negative for NSAE (section III, 7). Nineteen normal amniotic fluids were studied and compared to 28 abnormal amniotic fluids which were classified according to the main fetal abnormality into anencephaly (9), spina bifida (7), encephalocele (7), omphalocele (3). Finally, amniotic fluid was obtained on two separate occasions from a fetus which at termination of pregnancy had no NTD or structural malformations, but extensive skin damage. The latter

FIG. 2: Uncultured amniotic fluid cells.

Normal amniotic fluid:

a) Giemsa stain
b) Non-specific acid esterase (NSAE) stain

Amniotic fluid from a fetus with omphalocele:

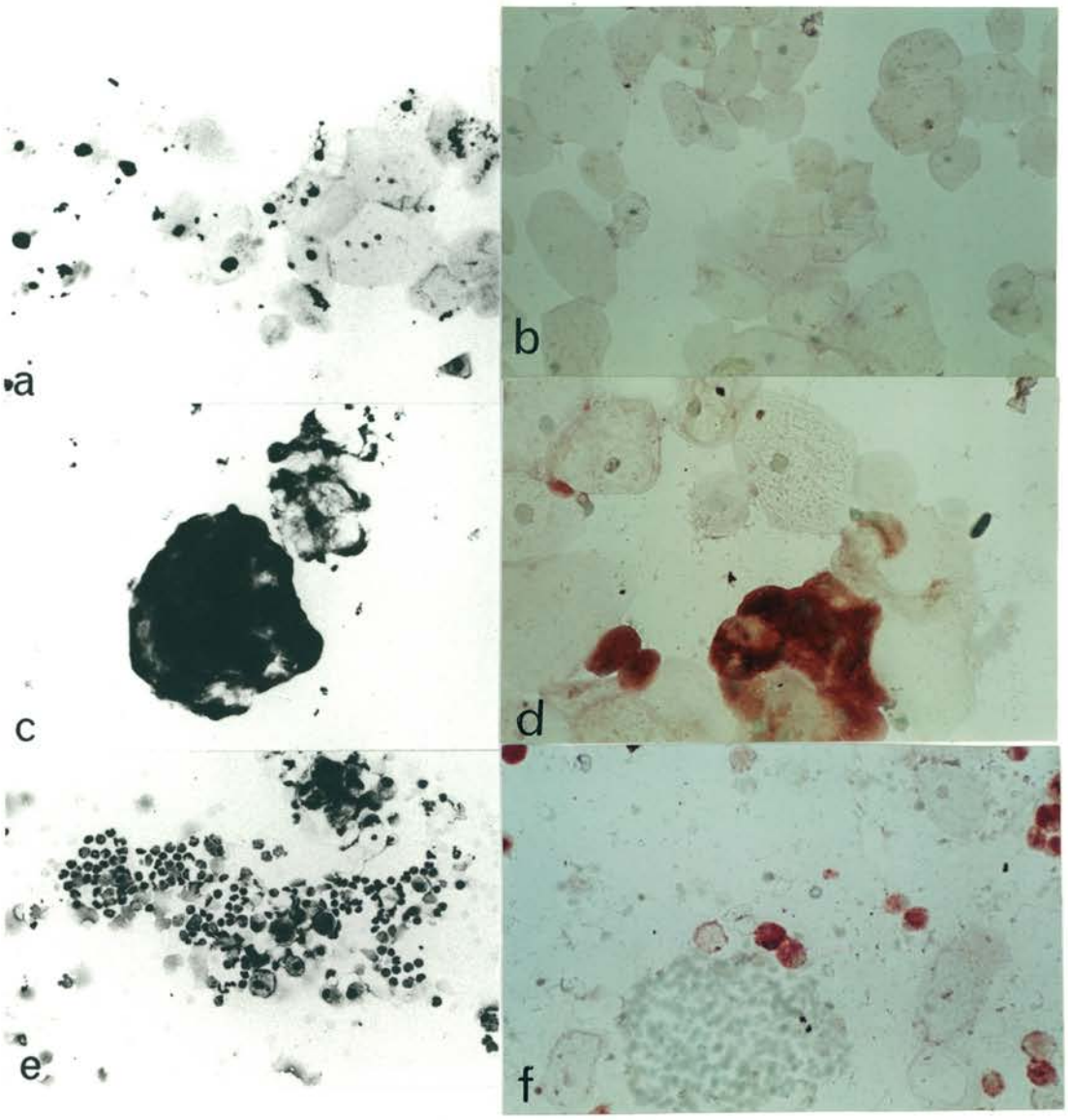
c) Giemsa stain
d) NSAE stain

Amniotic fluid from a fetus with a NTD:

e) Giemsa stain
f) NSAE stain

Magnification: (a, b, e, f) x 160

(c, d) x 256


a
b
c
d
e
f

two amniotic fluids were considered abnormal according to their AFP values (Brock, 1981).

Amniotic fluids from fetuses with a fetal lesion are often contaminated by a large number of RBC due to fetal bleeding. If this is the case, NSAE positive cells will be mainly of monocytic origin, ranging from monoblasts to macrophages. Other NSAE positive cells could be megakaryocytes, which are found in the fetal circulation by 10 weeks gestation (Hamilton et al., 1972; Wintrobe et al., 1974), and probably a few megaloblasts. Although megaloblasts are present in early gestation, by 16 weeks these have matured to normoblasts and erythrocytes (Hamilton et al., 1972; Hardisty and Weatherall, 1974). In amniotic fluids from fetuses with omphalocele there were both NSAE positive and negative cells, either isolated, or in association with the amorphous collagenous material (Fig. 2). The clusters of neural cells described by Chapman et al. (1981) were negative for NSAE stain but were associated with NSAE positive cells (Fig. 2).

In Tables 12 and 13 amniotic fluids are tabulated according to their number, classification (total cell count and RBC contamination), gestational age, amount of NSAE positive or negative cells, and outcome of pregnancy. It is interesting to note that amniotic fluid A-9 from a fetus with anencephaly, which had "normal" to "low" value of EARFC, also had a number of NSAE positive cells within normal values.

The number of NSAE positive and negative cells were scored against gestational age in Figs. 3 and 4 and the results, which

TABLE 12 NSAE IN UNCULTURED AMNIOTIC FLUID CELLS FROM NORMAL AMNIOTIC FLUIDS

No.	Classification	Gestation (weeks)	NSAE +	NSAE -	Outcome of Pregnancy
C-1	A/b	20	-	-	N.N.
C-2	a/b	18(s)	+	-	N.N.
C-3	a/b	16	-	-	N.N.
C-4	a/b	16	-	-	N.N.
C-5	a/b	17(s)	-	-	N.N.
C-6	a/b	18	-	-	Missed Abortion
C-7	A/B	18	-	-	N.A.
C-8	A/B	22	-	-	N.A.
C-9	A/b	19	-	-	Birth at 34 weeks Severe Pre-eclampsia
C-10	A/b	18	+	+	N.N.
C-11	A/b	20-21	++++	++	N.N.
C-12	A/b	20-21	+	+	N.N.
C-13	a/b	17	-	-	N.N.
C-14	A/b	18	-	-	N.N.
C-15	a/b	19	++++	+	N.N.
C-16	A/b	18	-	-	N.N.
C-17	A/b	22	-	-	N.N.
C-18	A/b	20	+	-	N.N.
C-19	A/b	18	-	-	N.N.

A - Total cell count > 70,000
a - Total cell count < 70,000
B - > 100,000 R.B.C.
b - < 100,000 R.B.C.
(s) - Gestational age given by scan.

\- < 25 cells
\+ 25-100
++ 101-200
+++ 201-300
++++ 301-400
+++++ > 401

N.N. - Normal newborn weight at birth > 2.5 k

N.A. - Not available

TABLE 13 NSAE IN UNCULTURED AMNIOTIC FLUID CELLS FROM ABNORMAL FETUSES

No.	Classification	Gestation (weeks)	NSAE+	NSAE-
I. NTD				
1. Anencephaly (A)				
A-9	a/B	19	++	++
A-11	a/b	18	+	++++
A-12	a/B	17	+++++	+++++
A-13	A/B	16	+++++	+++++
A-15		21	+++++	+++++
A-16	a/B	18	+++++	+++++
A-17	A/B	17	+++++	+++++
A-18	A/B	16	+++++	+++++
A-19	A/B	18	+++++	+++++
2. Spina bifida (S)				
S-3	A/b	20	+++++	+++++
S-4	A/B	18	+	++
S-5	a/b	18	-	-
S-7	A/B	17	++++	+
S-8	a/B	16	++	-
S-9	a/B	18	+	-
S-10	a/B	18	-	-
3. Encephalocele (E)				
E-1	A/b	19	+	-
E-2	a/b	25	+	-
E-3	A/B	18	+++++	+++++
E-4	a/b	17	-	-
E-5		20	+++++	-
E-6	a/b	20	++++	+
NO-2 Amniocentesis	a/b	20	-	+
Intact Sac	A/B	22	+++++	+
II. Omphalocele (O)				
O-2	A/b	21	++++	+++++
O-3	a/b	16	-	+
O-4	A/b	23	++	+
III. Other (Ot)				
Ot-1(a)	a/b	19	-	-
Ot-1(b)	a/B	20	+	-

See footnote Table 12.

FIG 3: Non-specific acid esterase (NSAE) positive cells in uncultured amniotic fluids.

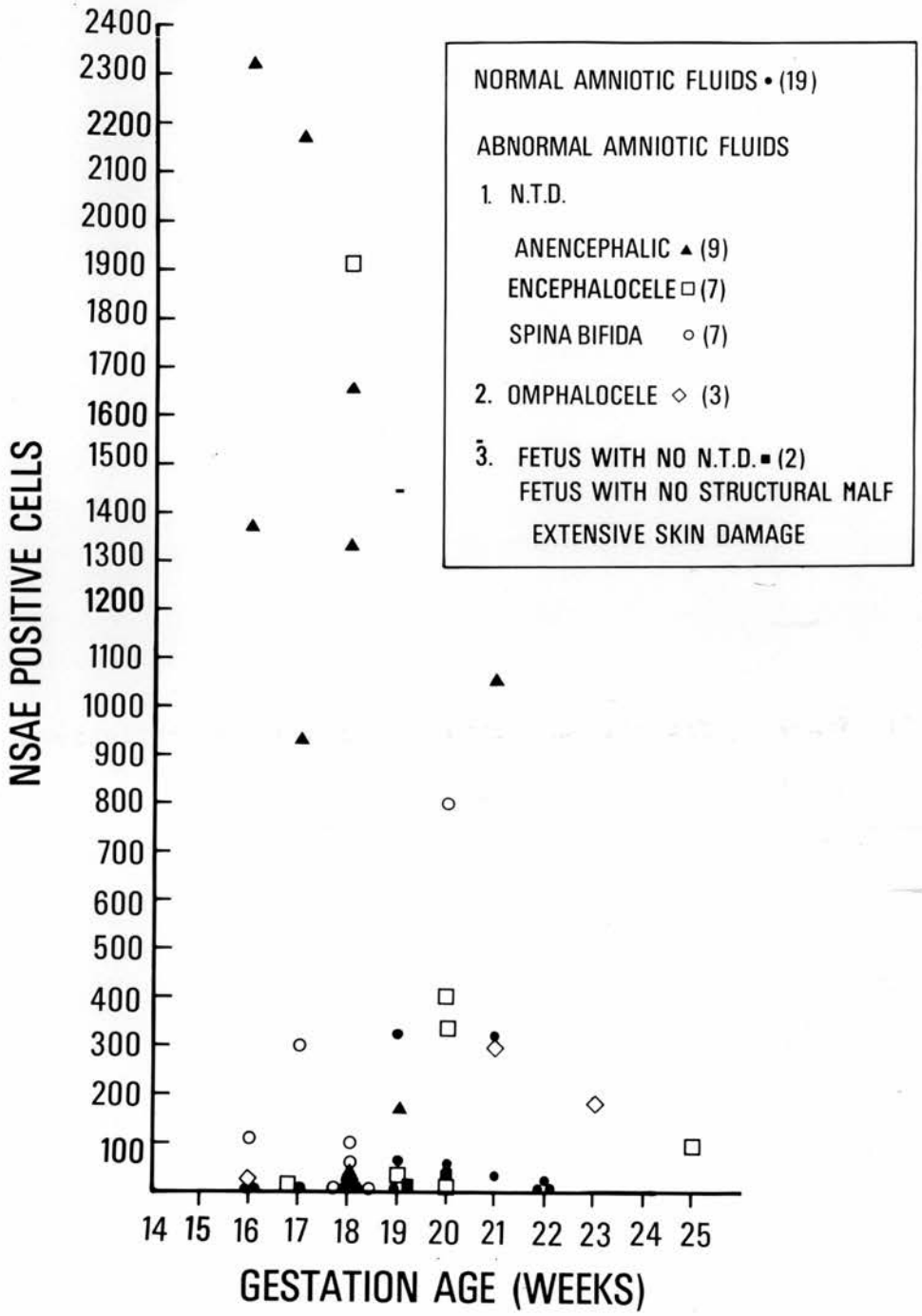
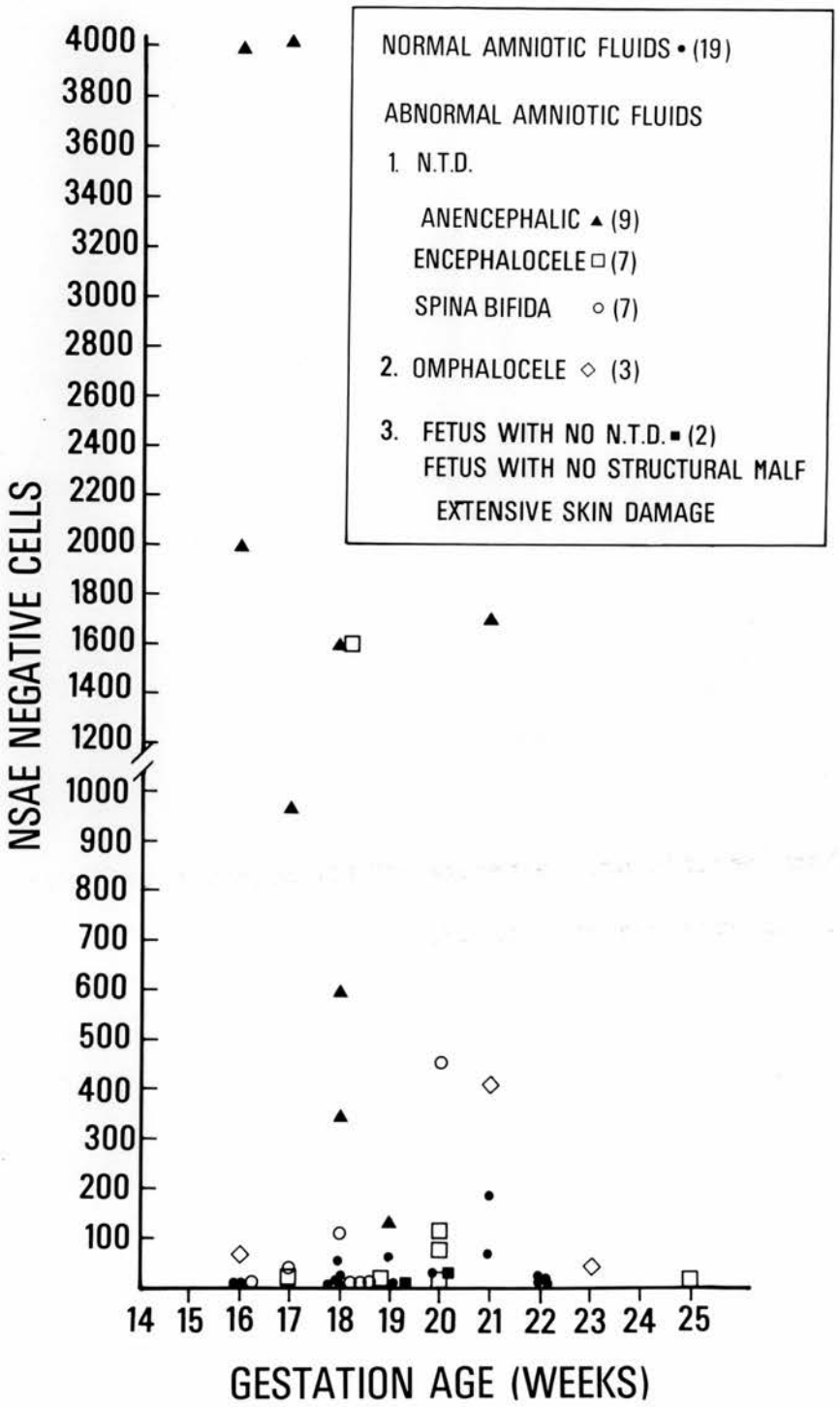

NSAE POSITIVE CELLS
2400
2300
2200
2100
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
14 15 16 17 18 19 20 21 22 23 24 25
GESTATION AGE (WEEKS)
NORMAL AMNIOTIC FLUIDS • (19)
ABNORMAL AMNIOTIC FLUIDS
1. N.T.D.
ANENCEPHALIC ▲ (9)
ENCEPHALOCELE □ (7)
SPINA BIFIDA ○ (7)
2. OMPHALOCELE ◇ (3)
3. FETUS WITH NO N.T.D. ■ (2)
FETUS WITH NO STRUCTURAL MALF
EXTENSIVE SKIN DAMAGE

FIG. 4: Non-specific acid esterase (NSAE) negative cells in uncultured amniotic fluids.


NORMAL AMNIOTIC FLUIDS • (19)
ABNORMAL AMNIOTIC FLUIDS
1. N.T.D.
ANENCEPHALIC ▲ (9)
ENCEPHALOCELE □ (7)
SPINA BIFIDA ○ (7)
2. OMPHALOCELE ◇ (3)
3. FETUS WITH NO N.T.D. ■ (2)
FETUS WITH NO STRUCTURAL MALF
EXTENSIVE SKIN DAMAGE
NSAE NEGATIVE CELLS
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
900
800
700
600
500
400
300
200
100
14 15 16 17 18 19 20 21 22 23 24 25
GESTATION AGE (WEEKS)

were statistically analyzed using the Mann-Whitney U non-parametric test (Siegel, 1956), are summarized in Table 14.

In amniotic fluids where there is no blood contamination or open fetal lesion, the NSAE positive cells will most probably be either placental macrophages (Fox and Kharkongor, 1970; Wood et al., 1978b) or trophoblastic cells (Wood et al., 1978b). Hoyes (1968) described variation in content of macrophages in amniotic fluids according to gestational age. Cutz and Conen (1978) found many macrophages in amniotic fluid samples up to 20 weeks of gestation, but less often during later gestation. In the present study there is an apparently normal distribution of the number of NSAE positive cells in normal amniotic fluids (Fig. 3). There is a low level at $\leq$ 16 weeks of gestation, the highest peak at 19 to 20 weeks of gestation and a low level again at 22 weeks.

The statistical analysis showed a very significant increase of NSAE positive and negative cells in amniotic fluids from fetuses with anencephaly but none in amniotic fluids from fetuses with spina bifida (Table 14). These results would agree with previous observations by Papp and Bell (1979) and Chapman et al. (1981). Amniotic fluids from fetuses with encephalocele had a significant increase only in NSAE positive cells but not of NSAE negative cells. However, there is one amniotic fluid (E-3) where the numbers of NSAE positive and negative cells are as high as some cases of fetal anencephaly. This suggests the possibility that the encephalocele was punctured during amniocentesis which has been reported before (Stirrat et al., 1979). Amniotic fluids from fetuses with omphalocele had a significant increase of NSAE negative cells but

TABLE 14 NSAE IN UNCULTURED AMNIOTIC FLUID CELLS

	NSAE Positive Cells (cells per 250 ml amniotic fluid)		NSAE Negative Cells (cells per 250 ml amniotic fluid)		Total Non-Squamous Cells (cells per 250 ml amniotic fluid)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Controls (19)	46	100	25	44	71	138
Anencephaly (9)	1231****	782	1694****	1442	2925****	2165
Spina bifida (7)	197	286	86	164	283	444
Encephalocele (7)	401*	689	257	587	658***	1265
Omphalocele (3)	167	143	170**	200	337**	329
Fetus with no NTD or structural malformation but extensive skin damage	17	22	12	13	29	35

* p = 0.05; ** p = < 0.05; *** p = < 0.02; **** p = < 0.002 (for a Two-tailed Test using the Mann-Whitney Test).

not of NSAE positive cells. The two amniotic fluids from the fetus which had no NTD or structural malformation but extensive skin damage, had values of NSAE positive and negative cells within the range of normal amniotic fluids.

B. Rapidly-Adherent Amniotic Fluid Cells

1. Morphology

The morphology of rapidly-adherent AFC has already been discussed in terms of the adherence alone or of their phagocytic properties (section I,C.1).

2. Adherence and phagocytosis

Three amniotic fluids from fetuses with anencephaly were tested for adherence and phagocytosis. Only non-squamous cells were scored due to the fact that squamous cells and FD I cells do not phagocytose (Table 3). Phagocytosis and phagocytic indices were calculated as previously described (section I, C.1). Cell adherence and phagocytosis are summarized in Table 15. The cell adherence of the 3 amniotic fluids after 20 min in culture ranged from 42 to 81% of the total cell adherence in 44 h. The phagocytic index ranged from 55 to 71%. However, in the three amniotic fluids, the highest cell adherence occurred within the first 10 min of culture.

TABLE 15 TIME OF ADHERENCE AND PHAGOCYTOSIS

	Controls (%) Adherence)			Phagocytosis (Phagocytic Index)		
	A-5	A-6	A-7	A-5	A-6	A-7
Gestation (weeks)	16	16	20	16	16	20
Minutes of adherence						
10	255 (68%)	316 (38%)	726 (68%)	75/132 (57%)	124/221 (56%)	228/313 (73%)
20	5 (69%)	38 (42%)	133 (81%)	2/7 (55%)	39/52 (60%)	12/26 (71%)
30	3	34	44	1/5	72/112	31/41
40	0	29	35	3/16	53/64	17/20
50	1	14	51	10/33	27/38	2/2
60	0	30	14	7/13	26/33	0/0
70	0	10	4	0/1	10/16	1/1
80	2	15	2	2/5	18/32	11/13
90	4	1	7	1/1	14/24	2/2
100	5	2	1	0/1	6/10	0/0
110	0	7	18	0/3	4/6	3/39
120	3	7	1	0/0	11/4	3/3
Sub total	278 (74%)	503 (60%)	1036 (97%)	101/217 (46%)	404/612 (66%)	310/460 (67%)
120 min - 44 h	98 (26%)	331 (40%)	29 (3%)	23/36 (64%)	84/266 (31%)	25/28 (89%)
Total	376 (100%)	834 (100%)	1065 (100%)	124/253 (49%)	488/878 (55%)	335/488 (69%)
Control Adherence 44 h	741	1345	1111	413/483 (85%)	406/665 (61%)	88/234 (38%)

$$\% \text{ Adherence} = \frac{\text{Adherent cells at x min.}}{\text{Total adherent cells at 44 h}}$$

$$\text{Phagocytic Index (\%)} = \frac{\text{Phagocytic cells at x min}}{\text{Adherent cells at x min}}$$

3. Fc receptors

Two amniotic fluids were studied (A-5 and A-8). Fc receptor avidity was measured as described previously (section IV, A.4). In both amniotic fluids 95% of the isolated adherent cells were EARFC positive. Phagocytosis of the sensitized RBC was observed. EARFC negative cells were either fibroblastoid or cells with branched processes. Cell clusters were EARFC negative but associated with EARFC positive cells. This would confirm the results on uncultured AFC from fetuses with anencephaly stained for NSAE stain (section IV, A.5).

4. Non-specific acid esterase (NSAE) stain

NSAE stain was performed on rapidly-adherent cells from 15 abnormal amniotic fluids: 8 from fetuses with anencephaly, 3 from fetuses with spina bifida, 3 from fetuses with encephalocele and 1 from a fetus with omphalocele. The number of NSAE positive or negative cells was extremely variable (Table 16). The highest number of both NSAE positive and negative cells was found in the amniotic fluids from fetuses with anencephaly. Most of the rapidly-adhering cells from fetuses with spina bifida were NSAE positive and very few cells adhered after 24 h in culture from the amniotic fluids of the fetuses with encephalocele. The only amniotic fluid from a fetus with omphalocele that was studied showed in the uncultured amniotic fluid an amorphous collagenous material with both positive and negative NSAE cells (Fig. 2). However, the cells migrating from the amorphous material after 24

TABLE 16 RAPIDLY-ADHERENT AMNIOTIC FLUID CELLS FROM ABNORMAL FETUSES

	NSAE Positive Cells		NSAE Negative Cells	
	Mean	S.D.	Mean	S.D.
NTD				
Anencephaly (8)	125	154	1169	2214
Spina bifida (3)	71	121	54	78
Encephalocele (3)	1	1	9	11
Omphalocele (1)	1	-	388	-

hours in culture were all NSAE negative.

5. Immunofluorescence for detection of tetanus toxin receptors

Fetal lung fibroblasts were consistently negative (Fig. 5).

Fetal brain in culture had two main cell types: small refractile cells with long processes which clustered on top of large epithelioid cells. Choi and Lapham (1976) studied human fetal brain cultures, with light microscopy, immunofluorescence and electron microscopy and showed that, during the first few days *in vitro*, the small cells were immature neurons and that the large epithelioid cells on which they were clustering were astrocytes.

In the present study, the small cells with processes were positive for tetanus toxin receptors, while the larger epithelioid cells were negative (Fig. 5). The positive cell membrane and processes stained bright green. However, cells on all the control fetal brain coverslips, including the one which had been treated with medium alone, had a mild yellow autofluorescence. Autofluorescence has been described previously in such organs as the brain, parts of organs such as renal convoluted tubules, in elastic tissue and in the granules of mast cells (Nairn, 1969). Mepham (1973) also described the autofluorescence of Nissl granules as golden yellow.

To establish the difference between the fluorescence of the labelled tetanus receptors and the mild autofluorescence of the fetal brain cells, the coverslips were unmounted, washed with saline,

FIG. 5: Immunofluorescence for detection of tetanus toxin receptors.

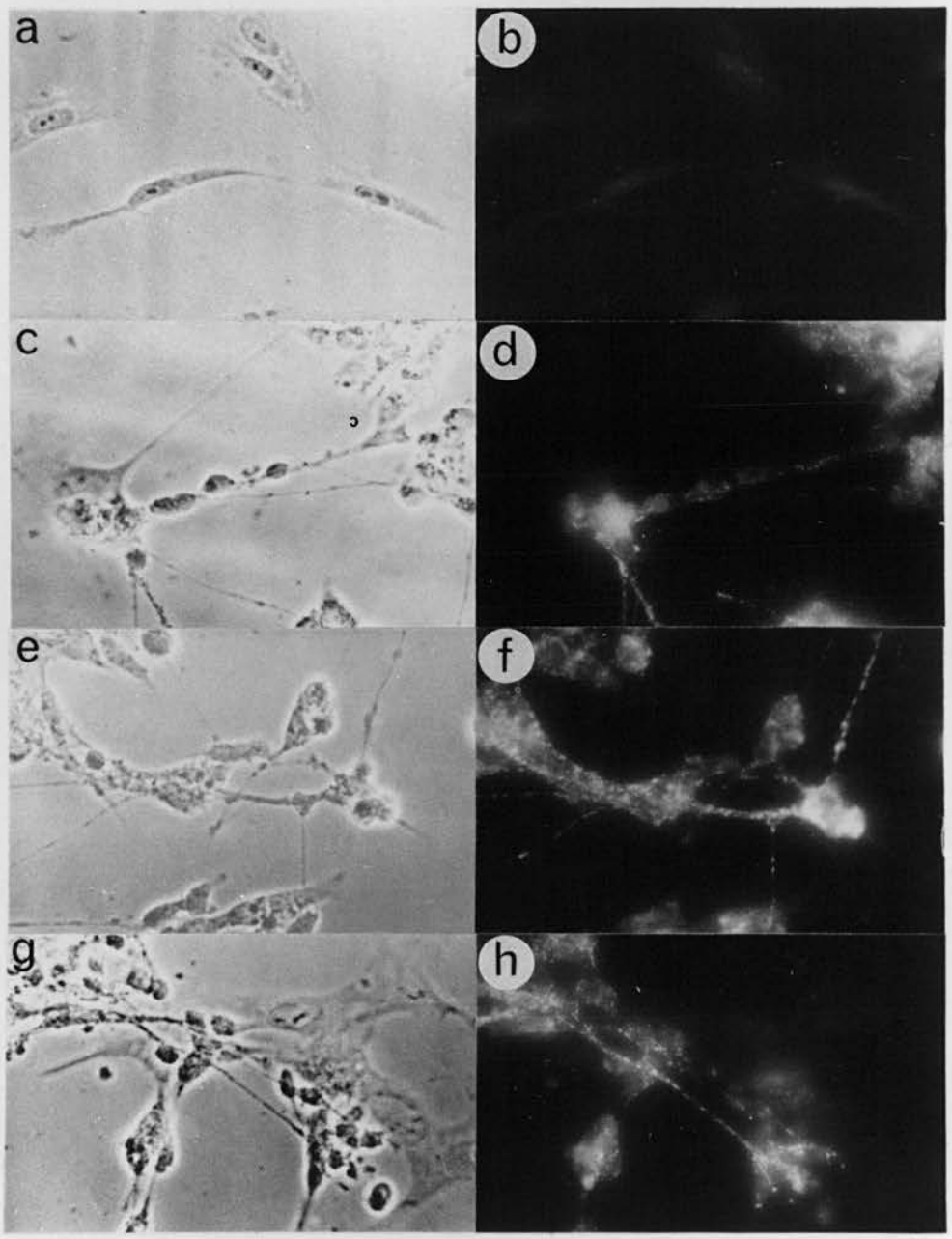
Fetal fibroblasts: negative for tetanus toxin receptors

a) Phase contrast
b) Immunofluorescence

Fetal brain: large epithelioid cells are negative for tetanus toxin receptors while small rounded cells with processes are positive

c,e,g) Phase contrast
d,f,h) Immunofluorescence

Magnification: x 243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g
h

counterstained with Evans blue 0.1% (Nairn, 1969) for 5 min and then washed in PBS for 10 min before remounting. With the counterstain, the autofluorescent cells now appeared red under the UV light, while the green fluorescence of the labelled tetanus toxin receptors remained. The control coverslips had only red cells while the coverslip that had been treated with tetanus toxin, equine antitoxin and the fluorescent-labelled antiserum showed cells with red cytoplasm and patchy bright green silhouettes or cells with red bodies and bright green processes.

This experiment proved that tetanus toxin could be used as a specific neuronal histologic marker in human fetal brain cells cultured for 6 days and obtained from a 12 week fetus.

Indirect immunofluorescence for detection of tetanus toxin receptors was tested in cells from three amniotic fluids from fetuses with a NTD; one with spina bifida and two with anencephaly (Table 17). Although consistently negative results were obtained with fetal lung fibroblasts, no positive controls were available due to the scarcity of fresh fetal brain and the necessity of using live cells for detecting tetanus toxin receptors. The possibility that the expression of tetanus toxin receptors required more than 20 h in culture was considered. Thus, rapidly-adherent AFC were cultured from 20 h to 4 days depending on the amount of amniotic fluid available.

The amniotic fluid from the fetus with spina bifida had very few rapidly-adherent cells, which were all negative for tetanus toxin receptors. In amniotic fluid A-4 one set of coverslips was counterstained while another was not. The cells on

TABLE 17 INDIRECT IMMUNOFLUORESCENCE FOR DETECTION OF TETANUS TOXIN RECEPTORS

Amniotic Fluid	Cell Number				Fluorescence			
	Control (Medium)		Experimental (T.T.)					
	Squamous	Non-Squamous	Squamous	Non-Squamous	Medium	T.T.-E.A.-Fl.	E.A.-Fl.	Fl.
S-2								
20 h in culture	4	1	7	24	-	-	-	-
A-4								
20 h in culture	4	1660	5	1463	-	-	-	-
3 days in culture	2	4154	9	65	-	-	-	-
A-5								
Polylysine 3 days in culture	14	43	15	33	-	+	+	+
No Polylysine 4 days in culture	10	372	11	516	-	-	-	-

T.T. - Tetanus Toxin E.A. - Equine Antiserum Fl. - Fluorescein labelled Immunoglobulin

the coverslips which had been counterstained, were stained red when viewed under UV light, thus they were negative for tetanus toxin receptors. In contrast, the cells on the coverslips which were not counterstained, and were treated with medium alone, were negative but those which had been treated with any antisera showed a fluorescent pattern different from fetal brain. The fluorescence was not present in the cell membrane or processes (as seen in fetal brain cells), but it was observed mainly in the cytoplasm surrounding the nucleus and forming small round vesicles or granules. This type of vesicular fluorescence was observed in most of the round macrophage-like cells of the amniotic fluid, while fetal lung fibroblasts were negative (Fig. 6). The coverslips containing cells with vesicular fluorescence were then unmounted and counterstained with Evans blue and remounted. The fluorescence disappeared and all the cells looked red under the UV light.

Recently, Raff et al. (1979) noticed a characteristic staining with most conjugates when used alone or with any of the antisera or normal sera. It consisted of the labelling of $\leq$ 5% of cells, all of which could be shown to be actively phagocytic. The staining was almost always inside the cells, lining small or large vacuoles, and was thus readily distinguished from the specific labelling of other cells. Raff et al. (1979) established that the labelling of phagocytic cells by the undigested conjugates was mediated by Fc receptors on these cells and concluded that they were macrophages. In all the rat tissues they tested, such as optic nerve, corpus callosum, cerebellum, cerebral cortex, leptomeninges, sciatic nerve and dorsal root ganglia, macrophages were present in no

FIG. 6: Immunofluorescence for detection of tetanus toxin receptors.

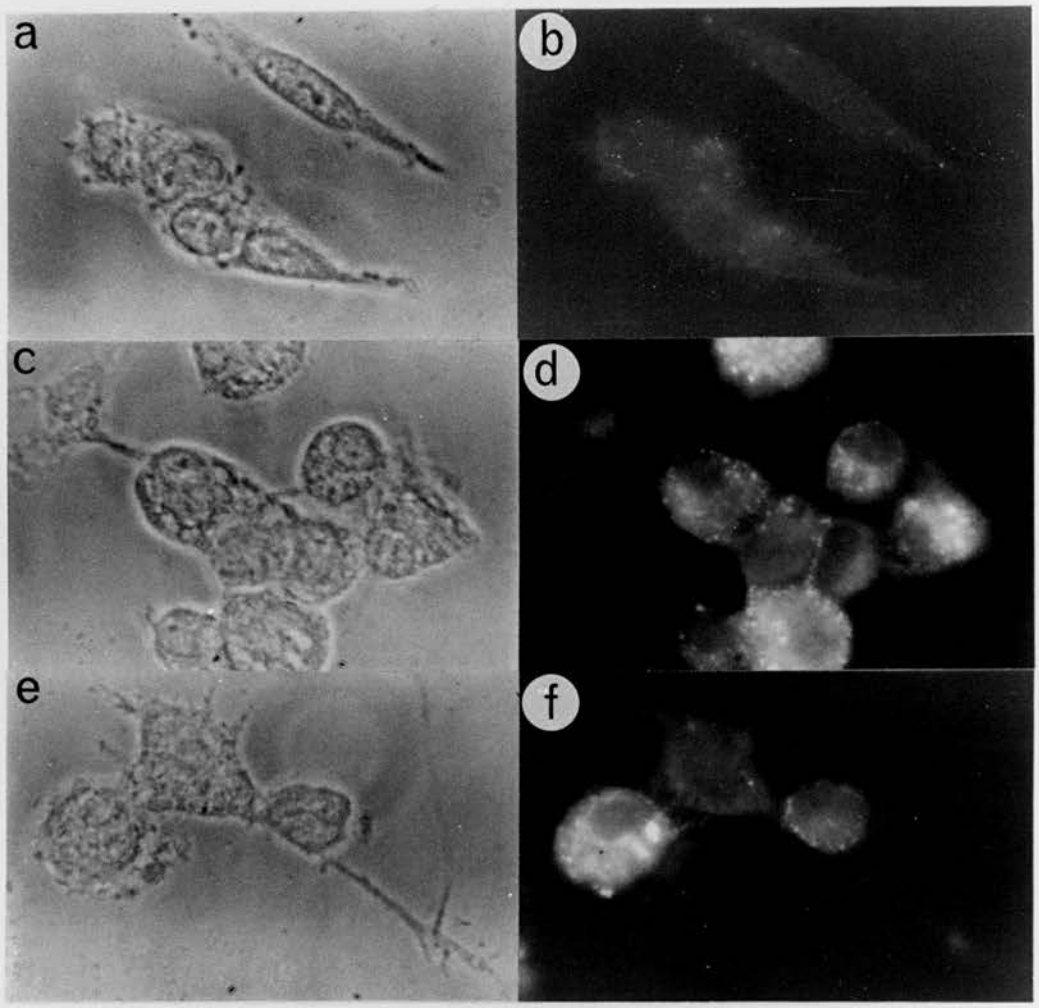
Fetal fibroblasts: negative for tetanus toxin receptors

a) Phase contrast
b) Immunofluorescence

Rapidly-adherent amniotic fluid cells from a fetus with anencephaly. Note the vesicular fluorescent pattern in the macrophage-like cells

c,e) Phase contrast
d,f) Immunofluorescence

Magnification: x 640


a
b
c
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e
f

more than 5% of cells. Kennedy (1980) cultured human fetal neural tissue from fetuses of 20 to 22 weeks gestation and found a maximum of 2.5% of macrophages in either dorsal root ganglia or spinal cord.

In amniotic fluid A-5, glass and polylysine-coated coverslips were used as substrate. The cells on the glass coverslips were negative for tetanus toxin receptors. The polylysine-coated coverslips had very few "unhealthy" looking adherent cells, some of which had long crooked processes. Wessels (1973) found that, by varying the concentration of calcium or serum in the medium, he could obtain crooked and bent axons, probably due to an increased adhesiveness. In the present study the medium used in glass or polylysine substrate experiments was the same, so the effect on the processes could only be attributed to the polylysine.

Immunofluorescent cells were observed on all coverslips coated with polylysine, even after counterstaining with Evans blue, except for the one that had been treated with medium alone. The positive staining of these cells is most likely to be due to the bad state of the cells leading to an increased permeability to the stain. Sternberger (1974) and Raff et al. (1979) point out that "unfixed" dead cells are penetrable to antibodies and stain non-specifically. The toxicity of the polylysine substrate for rapidly-adherent AFC is obvious when comparing the number of cells present on the two different substrates. There were slightly more squamous cells adhering to the polylysine substrate than to glass. Non-squamous cells were considerably affected with adherence of only 43 and 33 on polylysine compared to 372 and 516 on glass (Table 17).

The present results seem to suggest the absence of neurons in cultures of rapidly-adherent AFC from fetuses with a NTD. However, one should be cautious about the interpretation of the results. During the immunofluorescence procedure, many washes are required and unfixed cells tend to come off. In fact, the small cells with long processes which were positive in fetal brain, are the cells which are more loosely attached. Raff et al. (1979) pointed out this problem. They found a very low percentage (9 ± 5) of tetanus toxin positive cells in rat dorsal ganglion cells in culture and considered this figure artifactually low due to loss of neurons from the coverslips during washing.

6. Cinemicrography

The established criteria used to identify different cell types by cinemicrography are summarized in Table 18 and can be compared with the criteria used for fixed cell preparations (section I, C.1).

a) Short-term cultures of normal amniotic fluids

Four normal amniotic fluids (C-20 to C-23) were studied shortly after being plated. As has been previously described (Bowser-Riley, 1978; Cremer et al., 1981a), no cell adhesion was observed in any of the four amniotic fluids for up to 72 h in culture (Table 19). Examination of these cultures by phase microscopy showed that almost all the cells were squamous in morphology. Cinemicrography emphasized that these squamous cells floated in the medium and did not adhere, translocate or change their

TABLE 18 CHARACTERISTICS USED FOR CELL IDENTIFICATION BY CINEMICROGRAPHY

Epithelioid (E)	Adherent pavement sheets with ruffling membranes at periphery (Elsdale & Bard, 1974), isolated cells adhere poorly and bleb (Middleton, 1973).
Fibroblastoid (F)	Range between bipolar and fan-shaped with ruffling at leading edge (Elsdale & Bard, 1972), random movement (Gail, 1973).
Macrophages (M)	Rounded and refractile or flat and phase dark; they have extended pseudopodia and amoeboid movement (Petri et al., 1979), show phagocytosis (Meltzer et al., 1975) and are highly adherent (Gordon & Cohn, 1973).
Trophoblast	
Cytotrophoblast (Ct)	Mono or binucleated round or oval epithelioid cells with ruffling membrane (Valenti, 1965; Lueck & Aladjem, 1980; Aladjem & Lueck, 1981), microclastomatosis, rotational and translational movement of nuclei (Valenti, 1965). No divisions in short-term cultures (Valenti, 1965) but some divisions in long-term cultures (Lueck & Aladjem, 1980; Aladjem & Lueck, 1981).
Syncytiotrophoblast (St)	Large multinucleated giant cells with heavily granulated cytoplasm, ruffling membranes, syncytial strands and no division (Valenti, 1965; Lueck & Aladjem, 1980; Aladjem & Lueck, 1981). Rotational and translational movement of nuclei (Valenti, 1965). Projections, circular growth and sprouting (Lueck & Aladjem, 1980; Aladjem & Lueck, 1981).
Neural	
Neural general characteristics (N)	Numerous dendritic processes, active expansion and contraction of processes, microclastomatosis of preterminal and/or terminal knobs, branching and changing continually (Mulligan & Carnes, 1979).
Differentiating Neuroblastoma (Nb)	Development of processes by the cell body migrating away from a more stationary process (Booher et al., 1973).
Neuron (Ne)	Static cell body with extension of neurites showing fine filopodia; growth cone with similar ruffling to that of fibroblasts (Bray & Bartlett-Bunge, 1973).
Glial (G)	Branched cells with interconnecting processes, restless cell net (Lim et al., 1976), tug-of-war movement (Lim et al., 1976; Lumsden & Pomerat, 1951).
Microglia (Mi)	Cells with spiny processes and membrane movements resembling puffs of smoke (Pomerat, 1952).

TABLE 19 CINEMICROGRAPHY OF AMNIOTIC FLUID CELLS FROM NORMAL AMNIOTIC FLUIDS

Amniotic fluid	Time in culture before filming	Filming time	Adhesion +	Adhesion -	Cell No. in the field	Translocation +	Translocation -	Cell type
SHORT-TERM CULTURES								
C-20	2 h	19 h		-	+ + +		-	S
C-21	3 h	3 h		-	+ + +		-	S
C-22	1 h	27 h		-	+ + +		-	S
C-23	3 h 30'	19 h		-	+ + +		-	S
	72 h	4 h 30'	+		one		-	F
	76 h 30'	18 h 30'	+		+	+	-	F

Cell No. in the field: + sparse
+ + semi-confluent
+ + + confluent

Cell type: S - squamous
F - fibroblastoid

morphology (Fig. 7). In amniotic fluid C-23 a few cells attached to the substrate after 72 h in culture. Two fields were examined by cinemicrography; in the first, one adherent, elongated cell showed no evidence of movement or membrane ruffling; in the second, seven adherent cells in the field had fibroblastoid characteristics (Table 18), and there was ruffling over much of the periphery of the cell. The only migrating cell in the field was particularly noticeable for the high degree of ruffling at its leading edge.

b) Short-term cultures of abnormal amniotic fluids

There is an increase in cell adhesion after 20 h in culture of abnormal amniotic fluids. Cinemicrography showed that such adhesion could be transitory as cells would sometimes detach from the substrate. (In Tables 19, 20, 21 and 22, cultures where this happened are labelled as positive and negative for adhesion.) The cells chosen for cinemicrography were selected by phase microscopy and the medium was changed to remove RBC, squamous cells, dead cells and debris.

i) Amniotic fluids from fetuses with a NTD: Using phase microscopy short-term cultures of amniotic fluids from fetuses with a NTD showed the cell types described by Gosden and Brock (1978).

1) anencephaly: Five short-term cultures from amniotic fluids from fetuses with anencephaly were studied. These cultures had more adhering cells than cultures of amniotic fluids from fetuses with spina bifida and small cell clusters were frequently observed. Studies of rapidly-adherent cells from fetuses with anencephaly

FIG. 7: **Amniotic fluid cells present in primary cultures of normal and abnormal amniotic fluids.**

Short-term cultures:

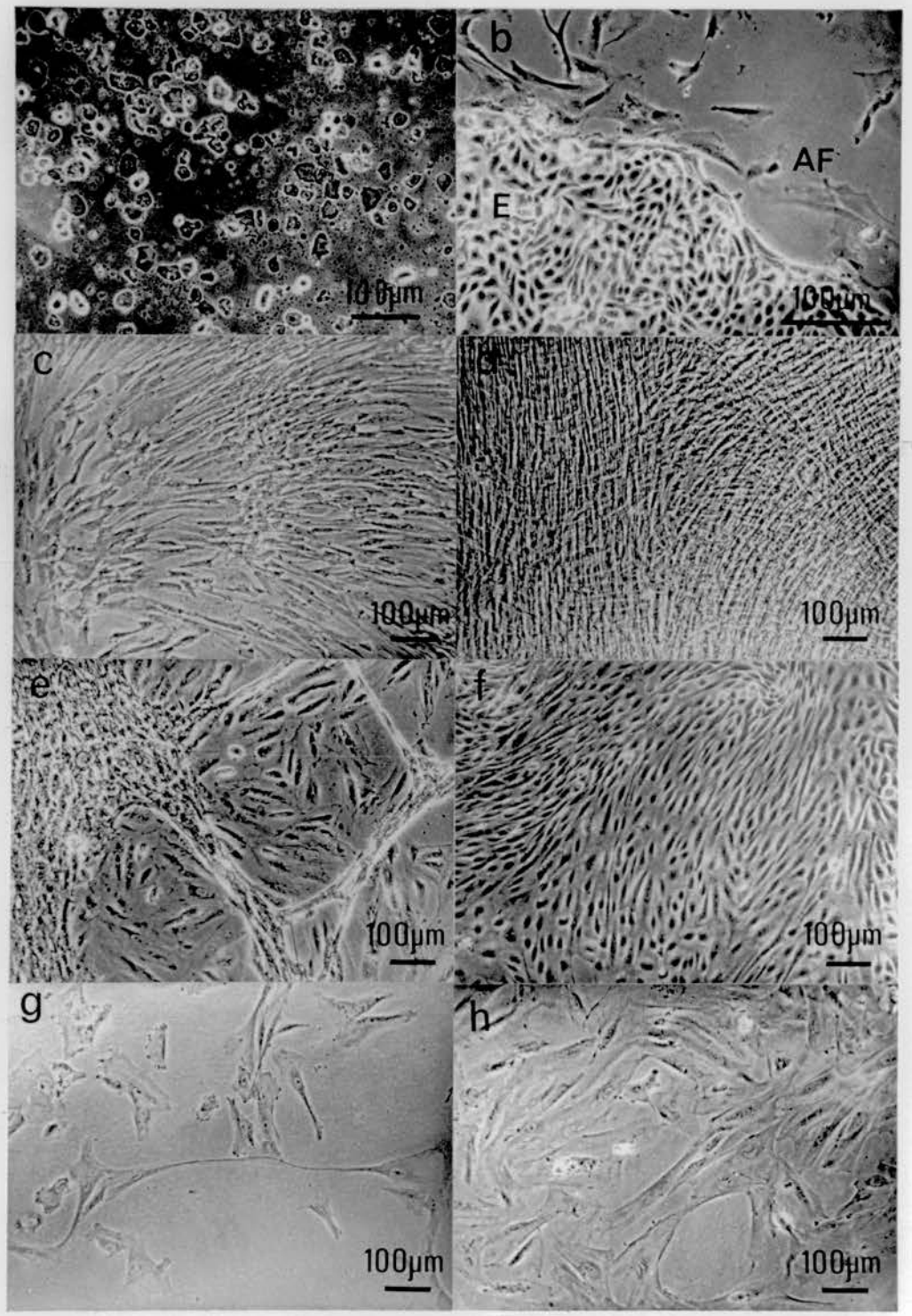
a) Squamous cells

Long-term cultures:

b) Epithelioid (E) sheet of cells with ruffling membrane at periphery. Amniotic fluid (AF) cells larger than E cells, pleomorphic and multinucleated
c) Fibroblastoid (F) cells, spindle in shape and forming parallel arrays
d) Orthogonal multilayer of fibroblastoid (F) cells

Cell patterns where a mixture of fibroblastoid (F) and epithelioid (E) cells are required:

e) Cable of fibroblastoid (F) cells spread across pavements of epithelioid (E) cells
f) Dermatoglyph pattern of epithelioid (E) cells
g) Amniotic fluid cell type (AF) forming syncytial strands
h) Amniotic fluid cell type (AF) with increased cytoplasmic granulation and circular growth


b
AF
E
100µm
c
100µm
d
100µm
e
100µm
f
100µm
g
100µm
h
100µm

investigating phagocytic properties, Fc receptors and non-specific acid esterase (NSAE) stain, have demonstrated that a varying proportion of these cells are macrophages. Most of the cell clusters present in amniotic fluids from fetuses with anencephaly were negative for Fc receptors and NSAE stain but were frequently associated with Fc or NSAE positive cells. For these reasons, cell clusters were preferentially chosen for cinemicrography which confirmed the presence of cells with macrophage and neural behaviour.

Cells which behaved like macrophages were observed in the five short-term amniotic fluid cultures studied (Table 20). In amniotic fluid A-1, a rounded refractile cell displayed the typical snail-like creeping movement attributed to macrophages (Petri et al., 1979). This macrophage-like cell was the only cell moving in the field and seemed to be inspecting the surface of a cell cluster where the cells displayed general neural characteristics (expansion and contraction of processes) and glial characteristics (branched cells with interconnecting processes) (Fig. 8). In A-2, phagocytosis was confirmed by cinemicrography; two cells phagocytosed latex particles (5.7 μm average diameter). The latex particles attached to the membrane and were engulfed. At this point the membrane ruffling stopped and reappeared shortly after, while the latex particles moved towards the cell nucleus (Fig. 8).

Cells with neural characteristics were observed in the five short-term cultures of amniotic fluids from fetuses with anencephaly (Table 20). These cells formed clusters of varying sizes and

TABLE 20 CINEMICROGRAPHY OF AMNIOTIC FLUID CELLS FROM FETUSES WITH ANENCEPHALY

Amniotic fluid	Gestation in weeks	Time in culture before filming	Filming time	Adhesion +	Adhesion -	Cell no. in the field	Translocation +	Translocation -	Cell type	Phagocytosis
Short-term cultures										
A-1	18	24h	15h	+		+ +	+	-	N,G,M	
		41h 30'	10h	+		+ +	+	-	N,G,M	
A-2	19-20	48h	6h	+		+ +	+	-	N,G	
		72h 30'	3h	+		+ +		-	N,G	+ (L.P.)
		75h 30'	1h 30'	+		+ +		-	G,M	+ (L.P.)
A-3	19-20	6h	14h	+	-	one		-	N	
	same	(24h	5h	+		+ +		-	S,G,M	
	field	(30h	15h	+		+ +		-	S,G,M	
		(51h	2h 30'	+		+ +		-	G,M	+ (L.P.)
A-4	18 same	(53h	17h	+		+ + +	+	-	N,G	
	field	(78h	15h	+		+ + +	+	-	N,G,M	
A-5	16	24h	22h 30'	+		+		-	N,G,M	
		66h	22h 30'	+	-	one		-	N	

Cell type: S - squamous
G - glial
N - neural (general characteristics)
M - macrophages

L.P. - latex particles

Cell no. in the field: + sparse
+ + semiconfluent
+ + + confluent

FIG. 8: Cinemicrography of amniotic fluid cells from fetuses with anencephaly. Short-term cultures.

a) Amniotic fluid A-1, 41 h in culture. Rounded macrophage-like cell (arrow) show snail-like creeping movement following the contour of the cell cluster (a - 0, a' - 5 h, a" - 9 h).

b) Amniotic fluid A-2, 75 h in culture: phagocytosis of latex particles.

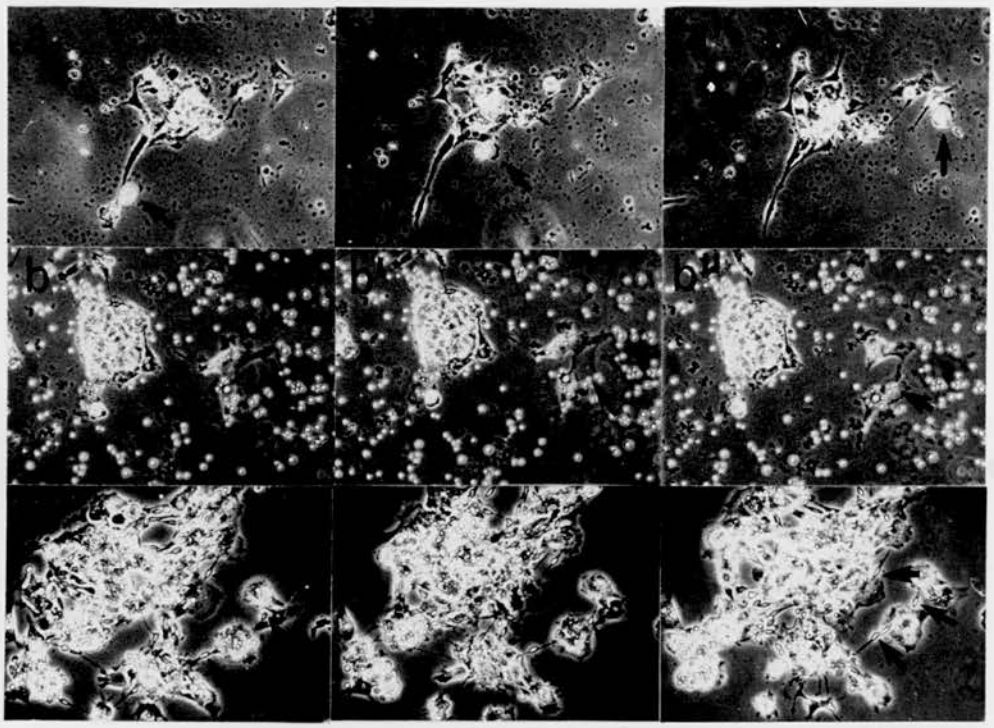
b (0) two latex particles adhere to the membrane (arrow).

b' (3') previous two latex particles are engulfed and another two latex particles adhere to the membrane (arrow).

b" (17') the four latex particles (arrow) have been engulfed and moved towards the centre of the cell; the membrane ruffling reappeared.

c) Amniotic fluid A-4, 78 h in culture. Tug-of-war movement (arrows) present in some bipolar cells of the cluster (c - 0, c' - 1 h, c" - 2 h 20').

Magnification: x 169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consisted of two cell types: large epithelioid cells and small cells with short processes which displayed either general neural characteristics or glial cell movement such as tug-of-war (Fig. 8). The small cells with processes were preferentially attached to the flat epithelioid cells. When isolated (A-3 and A-5) these small cells branched continually, struggled to remain attached to the glass coverslip, but finally rounded up.

There was no evidence of mitotic cell division in any of the short-term cultures of amniotic fluids from fetuses with anencephaly.

2) spina bifida: Two short-term cultures of amniotic fluids from fetuses with spina bifida were studied (S-1 and S-5). Fewer cells adhere in short-term cultures of amniotic fluids from fetuses with spina bifida than in those from fetuses with anencephaly (Table 2), so that only one or two cells were present in the field chosen for filming (Table 21). A cell with macrophage characteristics was observed in one amniotic fluid (S-5) and a cell with neural characteristics was observed in the other amniotic fluid (S-1). The neural cell behaved like a differentiating neuroblast, with elongation of the cell process as a result of the cell body moving away from the process. No phagocytosis of latex particles was observed in the neuroblast cell. The macrophage-like cell instead made contact with the second cell in the field which had fibroblastoid characteristics. At this point the membrane ruffling of the fibroblastoid cell stopped, the cell rounded up and the macrophage-like cell emitted pseudopodes as if trying to engulf it.

TABLE 21 CINEMICROGRAPHY OF AMNIOTIC FLUID CELLS FROM FETUSES WITH SPINA BIFIDA

Amniotic fluid	Gestation in weeks	Time in culture before filming	Filming time	Adhesion +	Adhesion -	Cell no. in the field	Translocation +	Translocation -	Cell type	Phagocytosis
Short-term cultures										
S-1	20	same field) 20 h	17 h	+		one	+		N,Nb	
		same field) 41 h	1 h 30'	+		one	+		N,Nb	- (L.P.)
S-5	18	46 h	20 h	+	-	two	+	-	M,F	+ (?)

Cell type: N - neural (general characteristics)
Nb- neuroblast
M - macrophage
F - fibroblastoid

L.P. - latex particles

Also see footnote Table 19.

However, phagocytosis was not clear (Table 21). Meltzer, Tucker and Breuer (1975) have described this cytostatic effect in BCG activated macrophages on neoplastic cells.

ii) Omphalocele (0-2): AFC from one fetus with omphalocele were filmed from 20 h to 9 days in culture (Table 22).

The amniotic fluid from the fetus with omphalocele had some amorphous material, probably collagen (Gosden and Ross, unpublished results). Although the amorphous material was not completely attached to the substrate, it seemed to be functioning as an explant from which cells were migrating. The cells had epithelioid morphology and moved very slowly in the field. They formed syncitial strands and processes broke away by microclastomatosis. The fragments exhibited rapid contraction and expansion, disappearing afterwards as debris. Such behaviour has been described in trophoblastic cells *in vitro* (Valenti, 1965). These cultured cells were negative for phagocytosis and non-specific acid esterase stain so they cannot be considered peritoneal macrophages. The probable origin of these cells is trophoblast, as has been suggested for some cells of long-term amniotic fluid cell cultures from normal fetuses (Priest *et al.*, 1977, 1978). No mitotic divisions were observed in up to 8 days of filming.

iii) NTD and omphalocele (NO-1): AFC from one fetus that had both a NTD and omphalocele were filmed from 2 days up to 7 days in culture (Table 22). Cells with macrophage or fibroblastoid characteristics were observed but none with neural properties. No mitotic cell divisions were observed.

TABLE 22 CINEMICROGRAPHY OF AMNIOTIC FLUID CELLS FROM A FETUS WITH OMPHALOCELE, AND A FETUS WITH OMPHALOCELE AND ANENCEPHALY

Amniotic fluid	Gestation in weeks	Time in culture before filming	Filming time	Adhesion +	Adhesion -	Cell no. in the field	Translocation +	Translocation -	Cell type	Phagocytosis
O-2	21	same field (20 h	8 days	+		+	+		S, Ct.	
		same field (9 days	4 h	+		+ +	+		Ct.	- (L.P.)
NO-5	20	48 h	4 days	+	-	+	+		M, F	+ (debris)

Cell type: S - squamous
M - macrophage
F - fibroblastoid
Ct. - cytotrophoblast

L.P. - latex particles

Cell no. in the field:
+ sparse
+ + semiconfluent
+ + + confluent

C. Long-term Amniotic Fluid Cell Cultures

1. Normal amniotic fluids

a) Morphology

Thirty primary long-term amniotic fluid cultures were carefully examined by phase microscopy (Table 23). The amniotic fluid cells had been maintained in culture from 20 to 60 days and were made available after karyotyping had been performed. The three types of AFC described by Hoehn et al. (1974) could be identified: E-epithelioid, F-fibroblastoid and AF-amniotic fluid "type". These cells were present in varying proportions and with a predominance of one cell type in some cultures.

Epithelioid cells formed pavement-like sheets. When the cultures were very confluent, the cells formed curved ridges resembling the dermatoglyphs described by Green and Thomas (1978) (Fig. 7).

Fibroblastoid cells formed parallel arrays (Fig. 7) and showed the typical spindle shape of confluent fibroblast cultures. Although amniotic fluid cultures are said to grow in monolayers (Martin, 1980), when cultures are grown for long periods of time they can become multilayered. Fibroblastoid cells formed the orthogonal multilayers described by Elsdale and Bard (1972) (Fig. 7). Mixtures of fibroblastoid and epithelioid cells showed a similar organization to that described by Bard (1979) in human embryonic

TABLE 23 DETAILS OF CULTURES IN WHICH CELL MORPHOLOGY WAS EXAMINED

No.	Gestation (weeks)	AFP Kiu/ml	Culture	Days in culture	Neuroblast morphology
ABNORMAL AMNIOTIC FLUIDS					
1. NTD					
A-10	18	143	P	16	+
A-13	16	175	P	35	+
A-14	20	124	P	20	+
A-20	18	101.4	P	41	+
A-21	19	206.5	P	32	+
S-3	20	97.5	P	12	+
S-6	18 (S)	55	P	30	+
S-7	17 (S)	63.5	P	19	+
S-8	16 (S)	56.2	P	32	+
S-10	18 (S)	68.4	P	34	+
2. Omphalocele					
O-1	18	186	S	70	-
O-2	21	154	P	38	-
O-5	17 (S)	281.6	P	35	-
3. NTD & Omphalocele					
NO-3	18	144.6	P	35	+
NO-4	19	265	P	36	+
NORMAL AMNIOTIC FLUIDS					
(30)	17-21	within normal limits	P	20-60	-
Fetal brain	15	-	P	14	+

(S) - gestation given by scan; P - primary culture; S - subculture.

kidney; here cables of cells originating from multilayered fibroblasts spread across pavements of epithelia (Fig. 7).

b) Cinemicrography

Five normal amniotic fluids which had been maintained in culture from 26 to 39 days were studied. The AFC were well attached to the substrate and the fields chosen for cinemicrography were either subconfluent or confluent (Table 24). The cells were identified according to the characteristics established in Table 18. Cinemicrography confirmed the classification of F cells as fibroblastoid and of E cells as epithelioid. Both cell types were seen to divide during periods of filming.

AF cells are said to be the main cell type in amniotic fluid cultures and are now considered to be trophoblastic in origin (Priest et al., 1979). In this study, AF cells were identified by the phase microscope according to the characteristics described by Hoehn et al. (1974). Cinemicrography of these cells confirmed their trophoblastic characteristics, and they could be divided according to previous studies into cytotrophoblast and syncytiotrophoblast (Table 18). Four long-term cultures of normal amniotic fluids which had AF cells were studied by cinemicrography (Table 24). All of these cultures contained cells with cytotrophoblastic or syncytiogrophoblastic characteristics and both types could be seen in a single field.

Cells with cytotrophoblastic characteristics consisted of small epithelioid cells which formed small clusters. Filming

TABLE 24 CINEMICROGRAPHY OF AMNIOTIC FLUID CELLS FROM NORMAL AMNIOTIC FLUIDS

Amniotic fluid	Gestation in weeks	Time in culture before filming	Filming time	Adhesion +	Adhesion -	Cell no. in the field	Translocation +	Translocation -	Cell type
Long-term cultures									
C-24	19	29 days	23h	+		+ +	+		Ct., St.
C-25	16 (s)	26 days	53h	+		+ +	+		E, Ct.
C-26	17	26 days	24h	+		+ + +	+		E, Ct.
		28 days	29h	+		+ +	+		Ct., St.
C-27	20	27 days	29h	+		+ + +	+		F
	same field	28 days	25h	+		+ + +	+		E, F
		29 days	24h	+		+ + +	+		E, F
		30 days	24h	+		+ + +	+		F
C-55	19	39 days	20h 30'	+		+ + +	+		Ct., St.

(s) - gestational age given by ultrasound

Cell no. in the field:
+ sparse
+ + semiconfluent
+ + + confluent

Cell type:
S - squamous
F - fibroblastoid
E - epithelioid
Ct.- cytotrophoblast
St.- syncytiotrophoblast

showed that these cell clusters did not make permanent adhesions compared with amniotic fluid cell type E which moved like a coherent pavement-like sheet of cells by means of a ruffling membrane at the periphery (Fig. 7). Cytotrophoblastic cells, on the other hand, seemed to have a type of locomotion that was intermediary between single cells and extended sheets (Albrecht-Buehler, 1977). Small cell clusters would move in opposite directions creating long syncytial strands (Fig. 7). Frequent mitotic divisions were observed in long-term cultures and, in some cases, the daughter cells moved in mirror image, changing direction in a symmetrical way (Albrecht-Buehler, 1977).

Syncytiotrophoblastic cells consisted of larger epithelioid cells, often multinucleated, with increased granulations in the cytoplasm; they seemed to move like a syncytial mass and were not observed to divide.

c) Non-specific acid esterase (NSAE) stain

In 11 normal amniotic fluids, cells attached to coverslips were available after karyotyping had been performed. The coverslips were stained for NSAE and at least 500 cells of each cell type present in one coverslip were counted. Total cell counts were not made because the amount of amniotic fluid that had been initially plated varied between 10 to 20 ml. The percentage of positive and negative cells was scored (Table 25). NSAE positive cells were observed in very low numbers either within E or AF cell colonies.

The fact that amniotic fluid cell colonies are not homogeneous has been described by Cremer et al. (1981) using antisera against

TABLE 25 PERCENTAGE OF NSAE IN AFC FROM LONG-TERM CULTURES OF NORMAL AMNIOTIC FLUIDS

Cell type	No. of amniotic fluids containing each cell type (one coverslip)	NSAE Positive Cells %	NSAE Negative Cells %
E-Epithelioid	4	0	100
F-Fibroblastoid	9	0	100
AF-Amniotic Fluid	7	2	98

intermediate filaments.

Cinemicrographic studies confirmed the trophoblastic behaviour of AF cell type cultures of normal amniotic fluids (vide supra). According to their characteristics, trophoblastic cells could be divided into cytotrophoblast and syncytiotrophoblast. Although Wood et al. (1978) found that a "significant" proportion of uncultured trophoblastic cells from placentae ranging from 12 to 16 weeks gestation were NSAE positive, the present results on cultured AF cells suggest that only a very low percentage (2%) of AF cells are positive for NSAE after more than 20 days in culture.

2. Abnormal amniotic fluids

a) Morphology

Amniotic fluid cultures from abnormal fetuses contained the same cell types described previously for normal amniotic fluids: E, F and AF cell types. Thirteen amniotic fluids were studied, 10 from fetuses with a NTD, 3 from fetuses with omphalocele and 2 from fetuses with both a NTD and omphalocele (Table 23).

Long-term amniotic fluid cultures from fetuses with a NTD, particularly those with anencephaly, grew extremely well as has been noted before by Nelson and Emery (1973). The long-term cultures of fetal brain and the abnormal amniotic fluids from fetuses with a NTD showed a similar morphology (Fig. 9). The cultures consisted of neuroblast-like cells (Choi and Lapham, 1976; Lim, 1980), which were small, rounded, very refractile, with long branching processes and formed clusters lying on top of large flat

FIG. 9: Live phase-contrast photographs demonstrating cell morphology and clustering of long-term amniotic fluid cell cultures from abnormal fetuses in comparison to a long-term culture of fetal brain.

a) Primary culture of fetal brain showing neuroblast-like cells above large flat glial epithelioid (G-E) cells

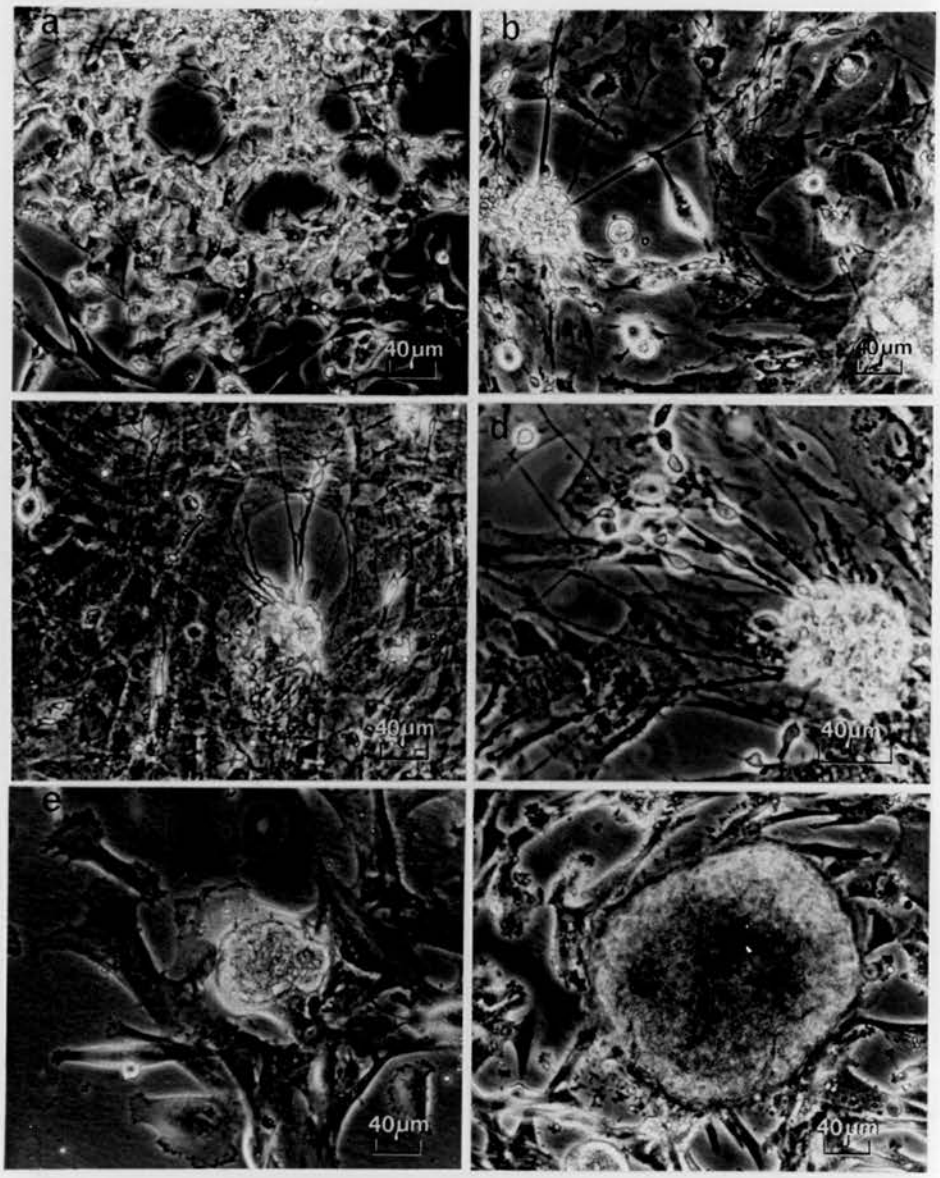
b) Primary culture from a fetus with iniencephaly and spina bifida (S-3). Neuroblast-like cells attached to the substrate of G-E cells

c) Primary culture from a fetus with anencephaly (A-13)

d) Primary culture from another fetus with anencephaly (A-20). Note the similarity with (c) at higher magnification.

e) Primary culture from a fetus with omphalocele (O-2)

f) Subculture from a fetus with omphalocele (O-1)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cells. In their studies of human fetal brain cultures, Choi and Lapham (1976) established by light microscopy, immunofluorescence and electron microscopy that, during the first few days *in vitro*, the small cells were immature neurons and that the large epithelioid cells on which they were clustering were astrocytes.

This neuroblast morphology has not been described in normal amniotic fluids (Hoehn *et al.*, 1974; Priest *et al.*, 1977a, 1978, 1979) and was not seen in any of the normal amniotic fluid cultures in this study (Table 23). The proportion of neuroblast-like cells varied in the different NTD amniotic fluids. Quantification was not attempted owing to the difficulty of counting clustered cells using phase microscopy.

A cytospin preparation performed on amniotic fluid S-3 (Fig. 2-e) which had obviously more neuroblast-like cells (Fig. 9-b), showed the clusters of small cells which have been described by Chapman *et al.* (1981) as neural cells (section IV, A.1). Chapman *et al.* (1981) found no evidence of differentiation in these uncultured neural cells either by electron microscopy or by the presence of the GFAP protein. However, cinemicrographic results (*vide infra*) proved that these undifferentiated neural cells can give rise to cells which behave like glial cells or neurons. Whether glial and neuronal cells originate from a common stem cell or from two different cell lineages needs further study (De Vitry *et al.*, 1980; Sueoka *et al.*, 1981).

Long-term cultures of rapidly-adherent GFAP positive cells after 18 days in culture have been described as epithelioid in morphology (Cremer *et al.*, 1981a), so in the present study it would have been very difficult to distinguish them from the E or AF cell

types. The number of neuroblast-like cells diminished with time in culture, being most probably overgrown by other cell types (Cremer et al., 1981a), and were not seen in subcultures (although occasionally a few very branched cells were found).

In long-term cultures of amniotic fluids from all three fetuses with omphalocele, the cells formed clumps, but unlike those amniotic fluid cells from fetuses with a NTD (vide supra), the cells had no processes (Fig. 9). In amniotic fluid 0-1, amorphous fibrillar material, probably collagen (Gosden and Ross, unpublished results), was either holding cells together or was being produced by the cells (Priest et al., 1977a, 1978). This material was observed in uncultured amniotic fluid, after 24 h adherence and in primary or secondary long-term cultures. In amniotic fluid 0-2, the clumping was not observed until later in culture and in 0-3 the clumps were much smaller and less numerous.

The two amniotic fluids from fetuses with both a NTD and omphalocele had some cells with neuroblast morphology, but not the clumping observed in the three amniotic fluids from fetuses with omphalocele.

b) Cinemicrography

Nine amniotic fluids from fetuses with a NTD were studied, 4 from fetuses with anencephaly, 3 from fetuses with spina bifida and 2 from fetuses with encephalocele. The two amniotic fluids from fetuses with encephalocele were compared to the cultures of cells obtained directly from the encephalocele.

i) Anencephaly: Four amniotic fluids from fetuses with anencephaly where the cells had been kept in culture from 10 to 42 days were studied.

The AFC were well attached and the fields chosen for cinemicrography were either subconfluent or confluent (Table 26). Examination of these cultures by phase microscopy showed clusters of neuroblast-like cells adhering to an underlying carpet of large epithelioid cells. The large epithelioid cells are most likely to be astrocytes (Choi and Lapham, 1976; Cremer et al., 1981a; von Koskull et al., 1981; Foucaud et al., 1982), but it is difficult to differentiate them from other epithelioid cells (E or AF) by their morphology alone. They will therefore be labelled as G-E (glial-epithelioid) cells.

Cinemicrography confirmed the presence of cells with neural behaviour in all 4 amniotic fluids studied. Amniotic fluid A-17 was maintained in culture for 10 days, and then cinemicrography was performed on five occasions up to 25 days in culture. Although the same region of the culture was studied, the fields were different on each occasion (Table 26). The neural cells differentiated during the 15 days of cinemicrographic study. Neuroblast-like cells clustered at the margin or on top of large G-E cells. The G-E cells moved in the field, carrying away the neuroblast-like cells and causing a change in direction or snapping of their processes. As has been described for differentiating neuroblastoma cells (Booher et al., 1973), the elongation of the neuroblast-like cell processes was due to migration of the cell body away from the more stationary process (Fig. 10). Neuroblast-like cells differentiated from apolar to bipolar and finally multipolar shapes. After 19 days in culture, the cells formed an intricate net of processes within the cluster (Fig. 10). At 24 days in culture, cells with distinct neuronal

TABLE 26 CINEMICROGRAPHY OF AMNIOTIC FLUID CELLS FROM FETUSES WITH ANENCEPHALY

Amniotic fluid	Gestation in weeks	Time in culture before filming	Filming time	Adhesion +	Adhesion -	Cell no. in the field	Translocation +	Translocation -	Cell type	Phagocytosis
Long-term cultures										
A-17	17	10 days	73h	+		+ +	+	-	N,Nb,G-E	
		13 days	20h	+		+ + +	+	-	N,Nb,G-E	
		19 days	31h	+		+ + +	+	-	N,Ne,G-E	
		24 days	24h	+		+ + +	+	-	N,Ne,G-E	
		25 days	6h	+		+ + +	+	-	N,Ne,G-E	
A-18	16	18 days	8h	+		+ +	+	-	Ne,G-E	
		19 days	15h	+		+ + +	+	-	Mi,G-E	
A-19	18 (s)	14 days	16h 30'	+		+ +	+	-	Mi,G-E	+(debris)
		15 days	83h	+		+ + +	+	-	Mi,G-E	+(debris)
		19 days	7h 30'	+		+ +	+	-	N,G-E	
		20 days	7h	+		+ + +	+	-	Ne, G-E	
A-20	18 same (	42 days	23h	+		+ + +	+	-	N,Ne,G-E	
	field(	43 days	19h	+		+ + +	+	-	N,Ne,G-E	
		45 days	23h	+		+ + +	+	-	N,Ne,G-E	
		46 days	50h	+		+ + +	+	-	N,Ne,G-E	

(s) - gestational age given by ultrasound

Cell type:
G-E - glial epitheliod
M - macrophage
N - neural (general characteristics)
Nb - neuroblast
Ne - neurons
Mi - microglia

Cell no. in the field:
+ sparse
+ + semiconfluent
+ + + confluent

L.P. - latex particles

FIG. 10: Cinemicrography of amniotic fluid cells from a fetus with anencephaly (A-17).

a) 10 days in culture:
(a - 0, a' - 1 h, a" - 13 h): small neuroblast-like cells migrate on top of glial-epithelioid (G-E) cells causing outgrowth, changes in direction and snapping of the processes.

a^{iii} - 25 h, a^{iv} - 37 h 30', a^{v} - 40 h): migration of neuroblast-like cells (arrow) from the cluster.

Magnification: x 169

b) 19 days in culture: small neuroblast-like cells within a cluster form an intricate net of processes.
(b - 0, b' - 14 h, b" - 19 h 30')

Magnification: x 281

c) 24 days in culture: process of neuronal clustering.

c-0 two multipolar neurons, lower neuron extends a neurite (arrow).

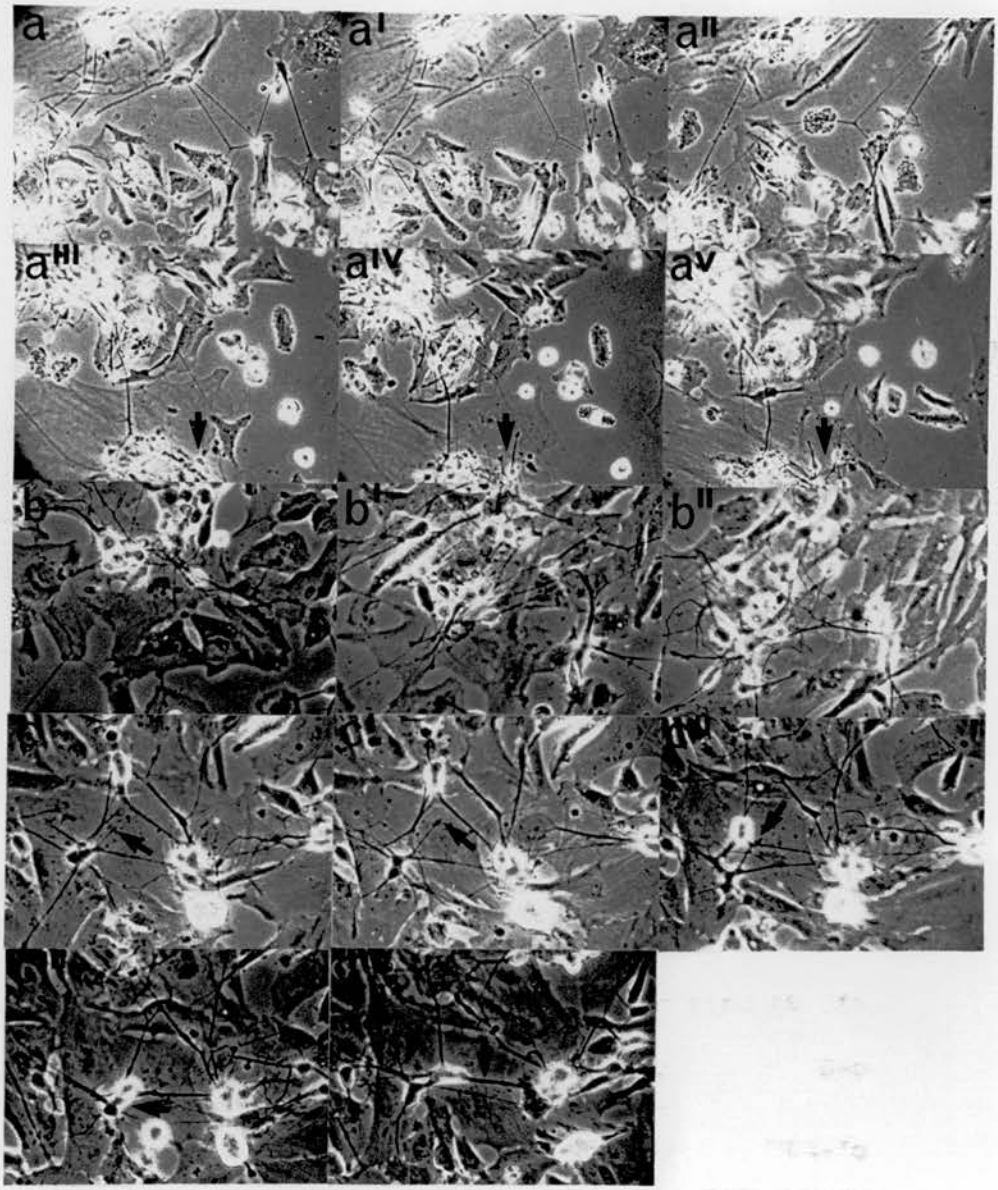
c'-50" outgrowth of the extended neurite (arrow).

c"-3 h 40' extended neurite (arrow) makes contact with a neurite of the upper neuron pulling both neurons closer together.

c^{iii} -4 h 45' contact of both neuronal cell bodies.

c^{iv} -6 h 30' straight thick bundles of neurites from both neurons have been formed (arrows).

Magnification: x 281


a
a'
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behaviour were observed. Unlike neuroblasts, neurones have a typical outgrowth of neurites, which move away from the static cell body by means of the ruffling membrane in the growth cone (Bray and Bartlett-Bunge, 1973). With increased time in culture, neurons become large and multipolar, forming small clusters from which the processes of two or more neurons form a thicker straight bundle of neurites (Fig. 10). In amniotic fluid A-20, the clusters of differentiating neurons increased in size with further culture (up to 43 days), forming a three-dimensional criss-crossing of neurites (Fig. 11).

Differentiation of AFC from fetuses with anencephaly was similar to that described for differentiating neurons of human fetal brain (Choi and Lapham, 1976) and embryonic rat brain in vitro (Sotelo et al., 1980; Abney et al., 1981; Raju et al., 1981).

Cells with neuronal characteristics were observed in all four long-term cultures from fetuses with anencephaly. The peculiar pumping mechanism observed by Pomerat (1952) was present in neuronal axons, as material from the cell body seemed to be forced distally all the way to the tip (A-18) (Fig. 11).

There are three glial cell types of the central nervous system: astrocytes, oligodendrocytes and microglia. Differentiating or differentiated astrocytes have a characteristic tug-of-war movement (Lim, 1980), which was observed in short-term cultures of amniotic fluids from anencephalic fetuses but was less evident in long-term cultures, probably due to the fact that a glial maturation factor is required to maintain the differentiated characteristics of astrocytes. Astrocytes will dedifferentiate in

FIG. 11: Cinemicrography of amniotic fluid cells from fetuses with a NTD. Long-term cultures.

a) Amniotic fluid A-20, 43 days in culture.
Large clusters of amniotic fluid neural cells showing three-dimensional criss-crossing of neurites.
(a - 0, a' - 5 h, a" - 9 h).

Magnification: x 281

b) Amniotic fluid A-18, 18 days in culture.
Neuron showing axonal outgrowth (arrow).
(b - 0, b' - 3 h, b" - 5 h).

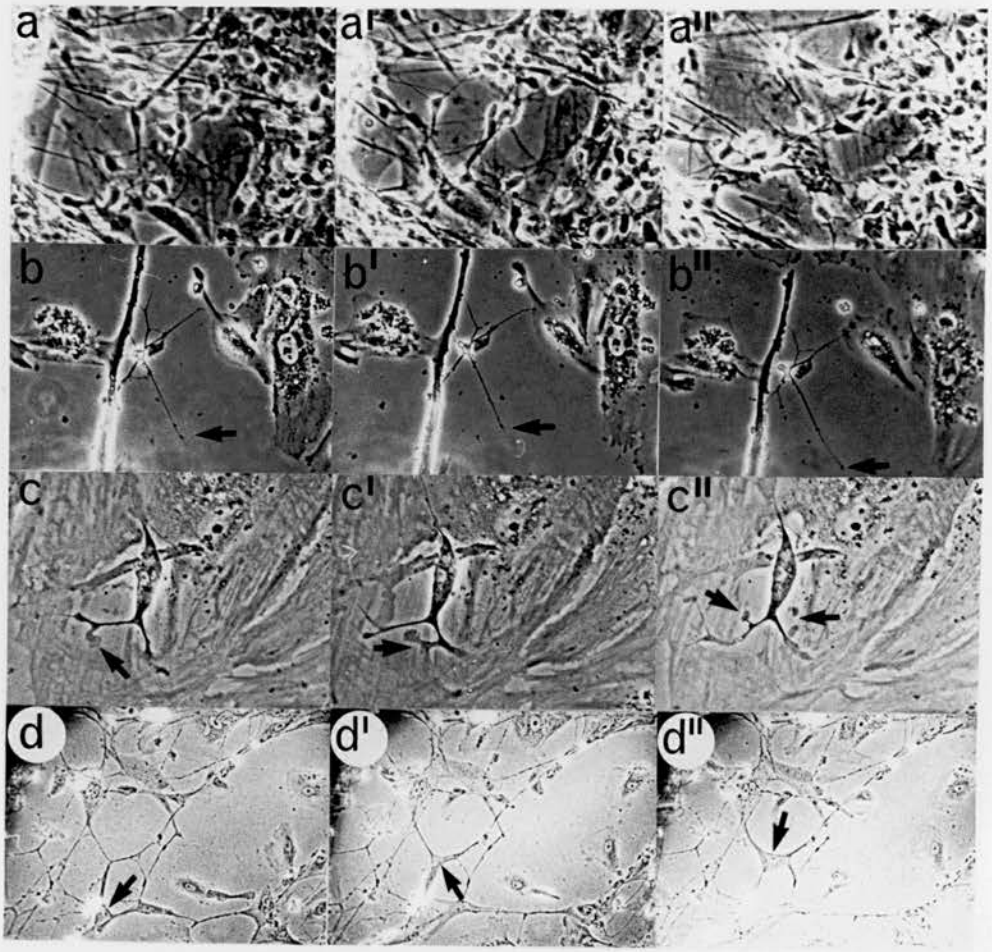
Magnification: x 169

c) Amniotic fluid A-18, 19 days in culture.
Microglial cell displaying very fine membrane movement simulating "puffs of smoke".
(c - 0, c' - 14', c" - 24').

Magnification: 281

d) Amniotic fluid S-10, 38 days in culture.
Glial-epithelioid (G-E) cell enveloping the bifurcating arms of a process with its ruffling membrane (arrow).
(d - 0, d' - 45', d" - 1 h 10').

Magnification: x 281


a
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the absence of this factor and will adopt a flat epithelioid morphology (Lim, 1980). Cell pulsation is a characteristic of both astrocytes (Lim, 1980) and oligodendrocytes (Lumsden and Pomerat, 1951), but was not observed in any of the cultures in this study. The third glial cell type, microglia, was observed in two long-term cultures of amniotic fluids from two fetuses with anencephaly (A-18 and A-19). Microglial cells are small, thorny-like cells with spiny processes which have a characteristic ruffling membrane that move simulating "series of puffs of smoke arising from multiple points" (Pomerat, 1952) (Fig. 11). Cinemicrography showed that some microglial cells were engaged in very active phagocytosis of cell debris.

Neuroblast-like or neuronal cells were never seen to divide. In contrast, G-E cells showed high mitotic activity and overgrew the cell clusters of differentiating neuroblasts.

ii) Spina bifida: Three amniotic fluids from fetuses with spina bifida, where the AFC had been kept in culture from 19 to 38 days, were studied (S-3 - S-5). Two showed cells with neural characteristics, being either neuronal or glial. In one amniotic fluid (S-5) only one coverslip was available and no neural cells were observed, instead epithelioid and trophoblastic cells were present (Table 27). As in amniotic fluids from fetuses with anencephaly, neuroblast-like or neuronal cells were seen in close association with G-E cells. In amniotic fluid S-4, an intricate net of cell processes and a few G-E cells were observed in the field, which was at the edge of a very confluent coverslip. One G-E cell followed a cell process, and at its bifurcation, the

TABLE 27: CINEMICROGRAPHY OF AMNIOTIC FLUID CELLS FROM FETUSES WITH SPINA BIFIDA

Amniotic fluid	Gestation in weeks	Time in culture before filming	Filming time	Adhesion +	Adhesion -	Cell no. in the field	Translocation +	Translocation -	Cell type
S-7	17	19 days	32 h	+		+ +	+	-	N,Ne,G-E, Ct.,St.,F
S-9	18	34 days	26 h	+		+ + +	+	-	F,E
		35 days	30 h	+		+ + +	+	-	Ne,G-E
		38 days	8 h	+		+ +	+	-	Ne,G-E
S-11	18-19	22 days	46 h	+		+ + +	+		E,Ct.,St.

Cell type: N - neural (general characteristics
G-E - glial-epithelioid
Ne - neurons
E - epithelioid
F - fibroblastoid
Ct. - cytotrophoblast
St. - syncytiotrophoblast

See footnote Table 26.

cytoplasm of the G-E cell with its ruffling membrane enveloped the two branches of the process (Fig. 11). Whether this G-E cell could have been a differentiating oligodendrocyte needs further study.

Few mitotic cell divisions of G-E cells were observed in long-term cultures of amniotic fluids from fetuses with spina bifida.

iii) Encephalocele: Two fetuses had an encephalocele. Cells obtained from the encephalocele and the amniotic fluid were studied at 6 and 13 days in culture (Table 28).

1) fetal encephalocele cells: Fetal encephalocele cells from fetus NO-2 adhered well to the substrate in contrast with fetal cells from fetus E-2 which adhered poorly. Using phase microscopy, the cell culture from the encephalocele cells of fetus NO-2 was very similar to the morphology of long-term cultures of AFC from fetuses with a NTD. Cinemicrography confirmed the presence of cells which had neural behaviour (either neuronal or glial) and also cells with fibroblastoid characteristics. Cinemicrography of the culture of the encephalocele cells from the second fetus (E-2) showed that the cells would adhere for short periods of time, branching and changing continually. Eventually the cells detached and seemed to have a higher cell-cell adherence than cell substratum adherence (Table 28).

2) amniotic fluid cells: Using phase microscopy and cinemicrography, cultured AFC from fetus NO-2 had the same characteristics as the cultured cells from its encephalocele. In

TABLE 28 CINEMICROGRAPHY OF FETAL AND AMNIOTIC FLUID CELLS FROM FETUSES WITH ENCEPHALOCELE

Cell No.	Cell source	Gestation in weeks	Time in culture before filming	Filming time	Adhesion +	Adhesion -	Cell No. in the field	Translocation +	Translocation -	Cell type
NO-2	Encephalocele	22	6 days	18 h	+		+ +	+	-	N, Ne, G-E
			13 days	22 h	+		+ +	+	-	N, Ne, G-E, F
	Amniotic fluid (Intact sac)		6 days	16 h	+	-	+	+	-	N, Ne, G-E, M
			13 days	22 h	+		+	+	-	S, N, Ne, G-E, F
E-2	Encephalocele	25	6 days	21 h	+	-	two		-	N
			13 days	24 h	+/-	-	+		-	N
	Amniotic fluid		6 days	21 h 30'	+		+	+		S, Ct.
			13 days	18 h	+		+	+		Ct., St.

Cell type: N - Neural (general characteristics)
G-E - Glial-epithelioid
Ne - Neurons
F - Fibroblastoid
M - Macrophages
Ct. - Cytotrophoblast
St. - Syncytiotrophoblast

See footnote Table 26.

contrast, cultured AFC from the second fetus (E-2) had only the AF cell type which displayed trophoblastic characteristics (Table 28).

c) Non-specific acid esterase (NSAE) stain

In ten abnormal amniotic fluids where coverslips were available, AFC were stained for NSAE. The amniotic fluid cells had been kept in culture from 14 to 46 days. The amount of amniotic fluid initially plated and the time of culture were so variable that total cell counts were not comparable. Instead, the cells in the coverslips were classified as fibroblastoid (F), glial-epithelioid (G-E) and neuroblast-like cells. Whenever possible, all the cells were counted and scored as positive or negative for NSAE. When the coverslips were very confluent, at least 500 cells of each cell type present in one coverslip were counted. Two coverslips, the first from an amniotic fluid from a fetus with spina bifida and the second from an amniotic fluid from a fetus with encephalocele contained no neuroblast-like cells (Table 29).

In order to compare the amount of NSAE positive cells in normal and abnormal amniotic fluids, either uncultured or cultured, the total proportion of NSAE positive cells was scored (Table 30). The highest proportion of NSAE positive cells was found in amniotic fluids from fetuses with spina bifida. However, the proportion of NSAE positive cells decreased with time in culture in both normal and abnormal amniotic fluids. In order to find out whether the NSAE positive cells were also decreasing in number, 7 abnormal amniotic fluids were studied uncultured at 24 h, 7 days and 14 days in culture. Fetal encephalocele cells were also studied and

TABLE 29 PERCENTAGE OF NSAE POSITIVE CELLS IN LONG-TERM CULTURES OF ABNORMAL AMNIOTIC FLUIDS

	Cell type	No. of amniotic fluids containing each cell type (one coverslip)	NSAE + (%)	NSAE - (%)
G-E	Glial-Epithelioid	10	2	98
F	Fibroblastoid	5	0	100
	Neuroblast-like	8	0	100

TABLE 30 PERCENTAGE OF NSAE POSITIVE CELLS IN AMNIOTIC FLUIDS

		Uncultured (cytospin preps.)			Rapidly-Adherent AFC (20-24 h Adherence)			Long-term Cultures ≥ 14 days in culture	
		Mean	S.D.		Mean	S.D.		Mean	S.D.
Normal amniotic fluids	(19)	38.34	28.01		N.T.	N.T.	(11)	0.64	1.29
Abnormal amniotic fluids									
NTD									
Anencephaly	(8)	43.5	17.37	(8)	20	20.74	(5)	0.4	0.55
Spina bifida	(7)	70.17	25.62	(3)	27.67	27.59	(4)	4.25	6.65
Encephalocele	(7)	65.71	37.48	(3)	2.67	4.62	(1)	0	0
Omphalocele	(3)	48.67	26.95	(1)	0	0		N.T.	N.T.

N.T. - not tested.

the results compared to those obtained with amniotic fluids.

The number of NSAE positive cells decreased significantly in all the amniotic fluids with the exception of the amniotic fluid from the intact sac of the fetus with cyclopia (NO-2) (Fig. 12). This amniotic fluid showed an increase in NSAE positive cells from 1 day to 7 days and from 7 to 14 days in culture. This might be explained as the effect of prostaglandins used in inducing termination of pregnancy.

The encephalocele cells from the same fetus also showed an increase in NSAE positive cells at 7 days in comparison to 1 day and then decreased again at 14 days in culture. Trapp et al. (1979) found a varying proportion of macrophages in aggregating cultures of mechanically dissociated rat brain. Macrophages were present in aggregates maintained in vitro for 4 days and 40 days but not in 19 and 26 day aggregates. They suggested that the reappearance of macrophages in the 40 day aggregates could be due to the presence of dormant cells which had been reactivated. However, this phenomenon was not observed in the encephalocele cells from the fetus with Meckel syndrome (E-2).

The NSAE negative cells increased in number with their culture in all the amniotic fluids from fetuses with anencephaly (Fig. 13), but decreased in amniotic fluids from fetuses with spina bifida, omphalocele or Meckel syndrome (Fig. 14). This could be explained by the initial cell density at plating which tends to be much higher in amniotic fluids from fetuses with anencephaly. The amniotic fluid cells and encephalocele cells from the fetus with cyclopia (NO-2) had the same growth pattern; NSAE negative cells

FIG. 12: Survival and proliferation of cultured non-specific acid esterase (NSAE) positive cells from abnormal amniotic fluids and fetal encephalocele cells.

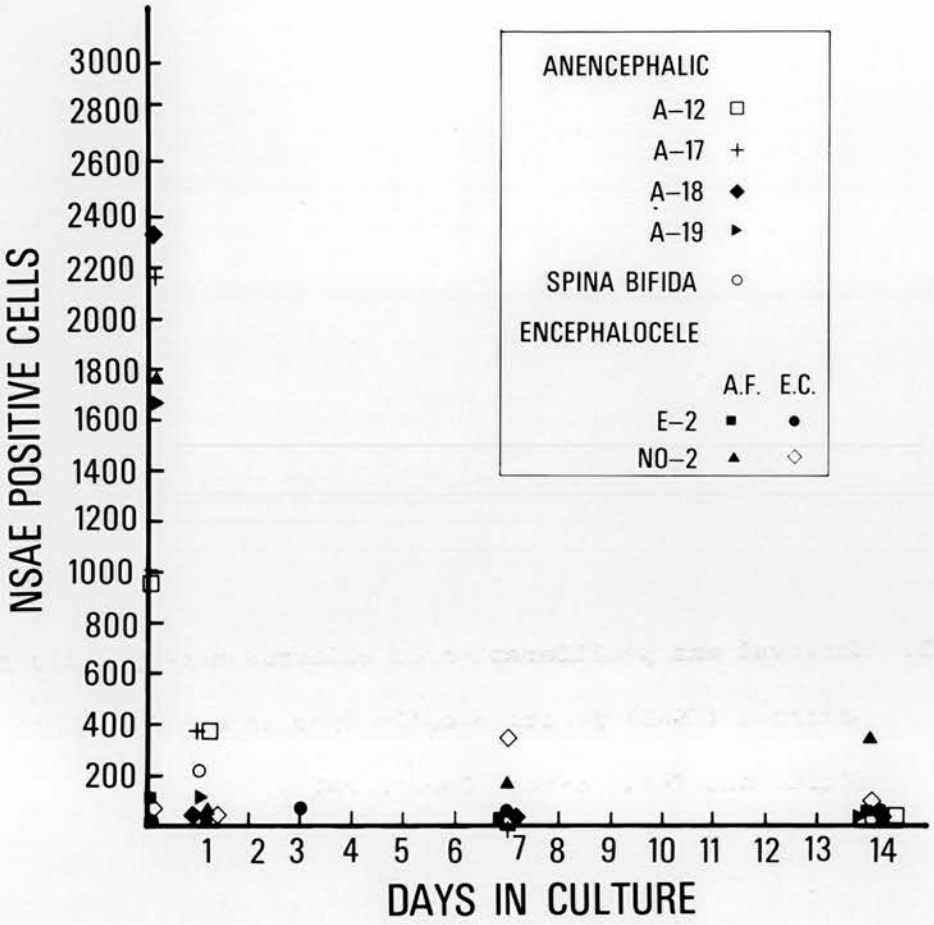
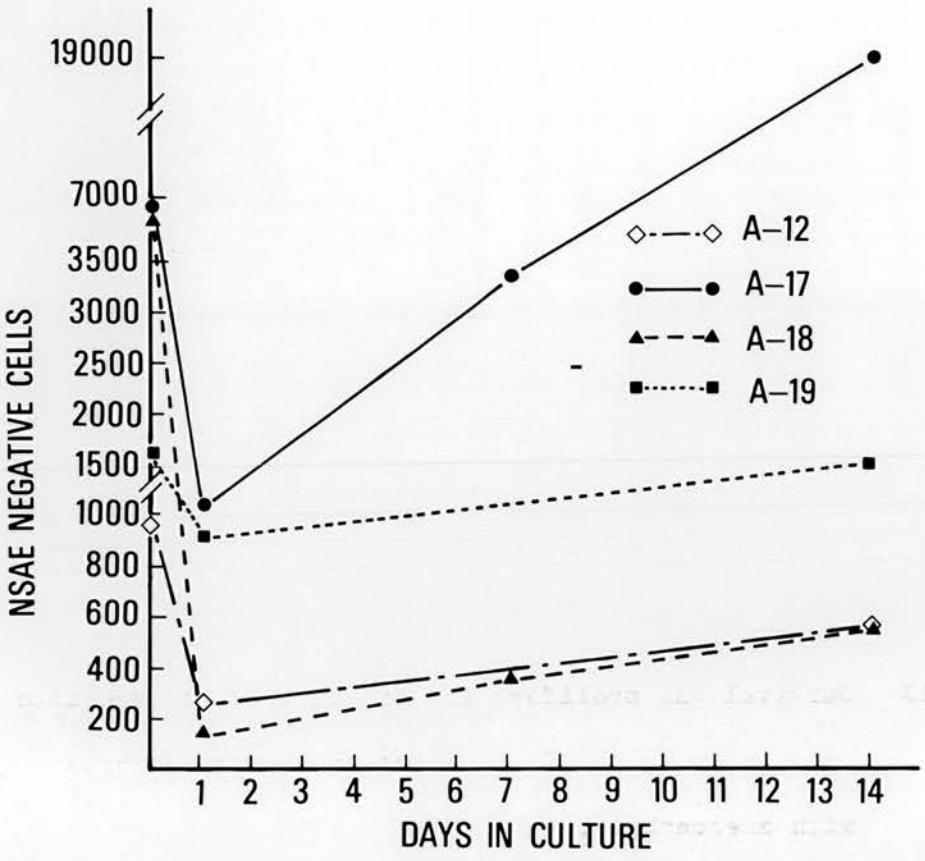

NSAE POSITIVE CELLS
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS IN CULTURE
ANENCEPHALIC
A–12
A–17
A–18
A–19
SPINA BIFIDA
ENCEPHALOCELE
A.F. E.C.
E–2
NO–2

FIG. 13: Survival and proliferation of cultured non-specific acid esterase (NSAE) negative cells from fetuses with anencephaly.


19000
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NSAE NEGATIVE CELLS
1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS IN CULTURE
A–12
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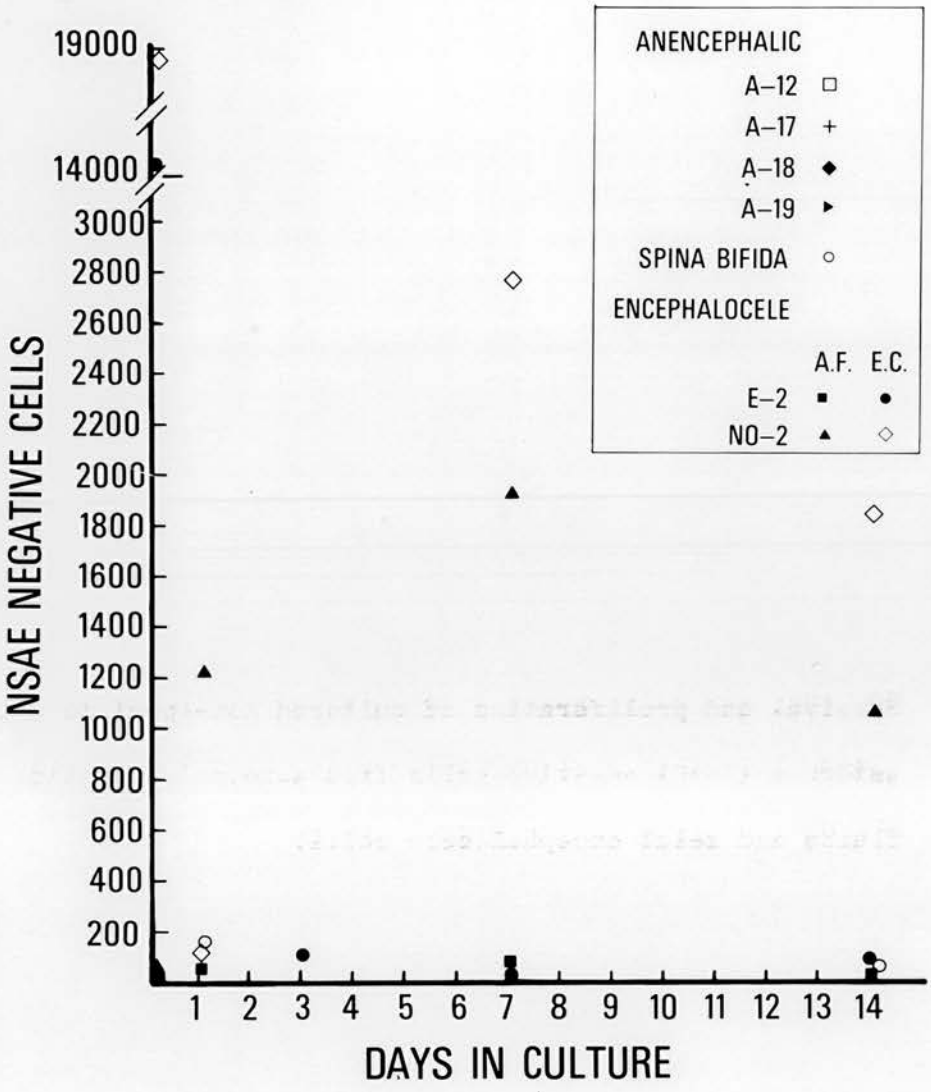
A–18
A–19

FIG. 14: Survival and proliferation of cultured non-specific acid esterase (NSAE) negative cells from abnormal amniotic fluids and fetal encephalocele cells.


ANENCEPHALIC
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SPINA BIFIDA
ENCEPHALOCELE
A.F. E.C.
E–2
NO–2
NSAE NEGATIVE CELLS
19000
14000
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS IN CULTURE

increased at 7 days in culture and decreased at 14 days in culture. These results would agree with those obtained by cinemicrography (section IV, C.2.3) where the same cell types were observed in both the culture of amniotic fluid and encephalocele cells from the fetus with cyclopia. Cinemicrography also confirmed the importance of cell-cell interaction. Neural cells adhere poorly to glass with an apparent increase in cell-cell adherence rather than cell-substratum adherence. Therefore the presence of different cell types will affect cell adhesion and cell growth.

D. Fetal Cells

1. Uncultured fetal cells

Fetal cells were studied in order to compare them to AFC from normal and abnormal fetuses. Cytospin preparations of uncultured fetal cells were stained with Giemsa and for the detection of NSAE.

Uncultured cells from fetal brain, spinal cord or cerebro-spinal-fluid (CSF) contained neural cells with the same size and morphology as the neural cells present in amniotic fluids from fetuses with a NTD. The main difference was that normal brain contained only small rounded neural cells, while the spinal cord from the fetus with anencephaly and the CSF from a fetus with spina bifida contained other cell types (Fig. 15). Duplicate cytospin preparations were stained for NSAE and the proportion of positive cells scored (Table 31). Examination of the spinal cord cells

FIG. 15: Uncultured fetal cells:

Spinal cord from a fetus with anencephaly (A-13)

a) Giemsa stain
b) NSAE stain

Cerebro-spinal-fluid from a fetus with spina bifida and Arnold Chiari syndrome (S-6)

c) Giemsa stain
d) NSAE stain

Note the activated appearance of the macrophages and the intensively positive NSAE staining of the lysosomes

Magnification: (a, b) x 256

(c, d) x 160

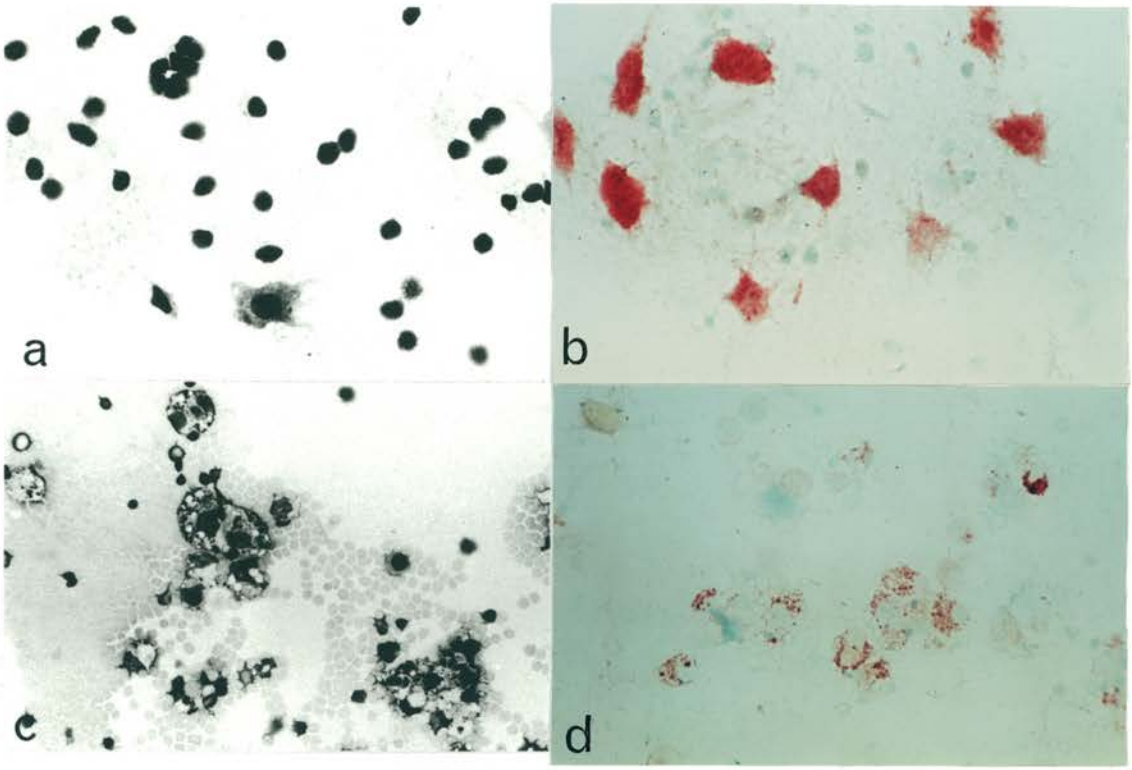
a
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TABLE 31 PERCENTAGE OF NSAE POSITIVE CELLS IN UNCULTURED FETAL CELLS

		Gestation (weeks)	NSAE Positive Cells %	NSAE Negative Cells %
Normal fetal brain		15	0	100
Encephalocele	E-2	25	0	100
	NO-2	22	0	100
Spinal cord	A-13	16	1.6	98.4
Cerebro-spinal-fluid	S-6	18	21	79

from the fetus with anencephaly showed that the small neural cells were NSAE negative but the larger cells were positive (Fig. 15) indicating a mononuclear origin. Mononuclear phagocytes of the central nervous system are NSAE positive (Oehmichen, 1978). An increase in macrophages has previously been described in sections of spinal cords from fetuses with anencephaly (Bell et al., 1981). Chapman et al. (1981) also found a large number of macrophages in sections of the neural lesion from fetuses with anencephaly.

CSF has not been studied before. The cytospin preparations of the CSF from the fetus with spina bifida contained not only a large number of macrophages but apparently also of activated macrophages. The macrophages contained many phagocytosed RBC and numerous granules which stained for NSAE (Fig. 15). CSF had the highest proportion of NSAE positive cells (Table 31). This suggests that a good proportion of the macrophages present in amniotic fluids from fetuses with a NTD must have leaked with the CSF into the amniotic fluid in a similar way to that suggested for acetylcholinesterase and AFP (Brock, 1980).

2. Fetal cells in culture

Fetal cells (and especially neural cells) were difficult to culture, due to their low viability.

Fetal lung fibroblasts were of spindle shape, aligned in parallel arrays, and were negative for tetanus toxin receptors (section IV, B.5) and for NSAE.

Fetal brain in culture displayed two main cell types: small refractile cells with long processes which were positive for

tetanus toxin receptors and large epithelioid cells negative for tetanus toxin receptors (section IV, B.5). Both cell types were NSAE negative.

V. DISCUSSION

The results of this study will be discussed on the basis of cell origin and their significance in antenatal diagnosis.

A. Macrophages in Amniotic Fluids

1. Origin

The origin of the macrophages present in normal and abnormal amniotic fluids is important for antenatal diagnosis. There are three possible sources of amniotic fluid macrophages: the open fetal lesion, fetal lung and placenta.

a) Open fetal lesion

In cases of omphalocele, peritoneal macrophages could be present in the amniotic fluid. In cases of NTD, macrophages might originate from the central nervous system (CNS).

i) Mononuclear phagocytes of the CNS: Oehmichen (1978) describes four types of mononuclear phagocytes of the CNS:

1) progressive microglia: they appear inside the cerebral parenchyma following various types of brain damage.

2) perivascular cells from the intracerebral vessels: these cells are characteristically located between the glial and endothelial basement membrane of intracerebral vessels.

3) free subarachnoidal cells: they appear in the subarachnoid

space with macrophage-like appearance.

4) epiplexus cells: these cells are located primarily on the choroidal epithelium inside the ventricular system.

After damage has been inflicted these cells cannot be differentiated from one another or distinguished from activated macrophages in other body regions.

The cytokinetic investigations of Oehmichen showed that mononuclear phagocytes in undamaged CNS and peripheral nervous system exhibit their own proliferative ability, even if only a very slight one. Under pathologic conditions this can be increased three to five-fold.

ii) Glass-adhering cells of the CNS: Oehmichen (1978) also studied glass-adhering cells after intracerebral glass implantation in rabbits, and observed three different types:

1) mononuclear foam cells: phagocytic and positive for Fc receptors.

2) polynuclear giant cells: phagocytic and positive for Fc receptors.

3) large spindle cells: non phagocytic and negative for Fc receptors.

Papp and Bell (1979) and Chapman et al. (1981) suggest that the macrophages in amniotic fluids from fetuses with a NTD come from the fibrovascular membrane covering the lesion. In addition, Bell et al. (1981), in their study of abnormalities of the spinal meninges in fetuses with anencephaly, found that the leptomeninges

were excessively vascular and contained many active macrophages. It is interesting that macrophage activity was less striking, although greater than normal, in three cases where no heterotopic nodules of apparently immature neural tissue surrounded the pia mater. This could be one reason for the varying number of NSAE positive cells in cases of anencephaly. In the present study activated macrophages were also found in the CSF of a fetus with spina bifida and Arnold-Chiari syndrome.

b) Lung

The second possible source of macrophages in amniotic fluids from fetuses with anencephaly might be fetal lung. Emery (1974) observed that human lungs are free of lymphoreticular aggregates at birth which appear only later in life as a response to environmental factors. In lungs of children dying at the time of birth or within a few days thereafter, these types of aggregates occurred in only two conditions: intrauterine infection and infants with meningocele or anencephaly. Whether these cells are the result of an antigenic stimulus is not known, but it is interesting that lung has been suggested as the organ by which macrophages are removed from the body (Nicol et al., 1958). A recent study using electron microscopy (Tyden et al., 1981) has shown that, in cases of amniotic fluids from normal fetuses at mid-trimester, there are virtually no cells in the amniotic fluid from the respiratory tract, urinary bladder or amniotic membranes. It would be interesting to repeat these studies in cases of fetuses with a NTD.

c) Placenta

The third source of macrophages in amniotic fluids might be placenta.

i) Normal amniotic fluids: Macrophages have previously been described in normal uncultured amniotic fluids (Hoyes, 1968; Casadei, 1973; Cutz and Conen, 1978; Papp and Bell, 1979; Chapman et al., 1981). They seem to appear at that stage in gestation when placental macrophages are more numerous in placenta (Fox, 1978). Stimulation of macrophages with hormones such as oestrogens has been described (Nicol et al., 1958).

ii) Amniotic fluids from fetuses with anencephaly: The present study agrees with the studies of Papp and Bell (1979) and Chapman et al. (1981) that there is an increase in macrophages of amniotic fluids from fetuses with anencephaly. Ten Berge (1965), in his studies of placentae from fetuses with anencephaly, found an increase of placental macrophages in 50% of the placentae, while many also showed an increase of cytotrophoblastic cells. In contrast, Fox (1978) found many normal placentae from fetuses with anencephaly; a few were oedematous and a small proportion had villous immaturity. Papp and Bell (1979) also found that amniotic epithelium in cases of fetal anencephaly had an increased macrophage layer with quantities of PAS positive material, fat droplets and haemosiderin. Although recent reports (Tyden et al., 1981; Bergström, 1979) of normal pregnancies have indicated that there is no exfoliation from amniotic epithelium, these studies have not been extended to cases of congenital malformations such as NTD. Both studies agree that normal amniotic epithelium shows frequent intercellular openings.

However, intercellular openings are apparently not required for cell migration, as has been recently demonstrated by Russo et al. (1981) in their studies of polymorphonuclear (PMN) leukocyte migration through human amnion membrane. Using transmission electron microscopy they found that PMN attached to the epithelial surface, infiltrated between intercellular junctions (migrating around or through tight junctions and hemidesmosome attachment), penetrated the basement membrane and migrated through dense collagenous stroma. Some amniotic fluid cells in long-term cultures are thought to come from placenta, either stroma or trophoblast (Priest et al., 1979), so there is no reason why placental macrophages should not reach amniotic fluid.

iii) Amniotic fluids from fetuses with Rhesus isoimmunization: An increase of amniotic fluid macrophages has also been described in pregnancies where there was severe Rhesus isoimmunization (Sutherland et al., 1975). Placental macrophages are increased in most cases of Rhesus isoimmunization and this could be due to placental immaturity or oedema (Fox, 1978). It is interesting that Pasquinucci et al. (1969) in their study of uncultured amniotic fluids found that 17 out of 99 showed the presence in the sediment of unclassified cells and abundant cellular debris. Of these 17 cases, 15 were from patients carrying fetuses with severe Rhesus isoimmunization, suggesting this could indicate a stage of "fetal suffering".

iv) Amniotic fluids from high risk pregnancies: Cells described as fetal distress cells by Gosden and Brock (1978b) adhere rapidly to glass and have phagocytic properties (Medina-Gómez, 1979). These

cells have been associated with spontaneous abortion, low birth-weight and pre-eclampsia (Gosden and Brock, 1978b).

Recently, Lauslahti and Ikonen (1980), in their studies of 468 placentae, found that 75% of the infants delivered before 32 weeks and nearly 50% between 32 and 37 weeks had an abnormal placenta. Ablation of the placenta in particular, but also inflammatory changes and small fibrous placentae were found in close association with premature births. A large "embryonal-persistent" placenta was found in one of two malformed infants. Ladermacher et al. (1981) have also found that circumvallate placentae were associated with perinatal death, premature delivery and congenital malformations.

It is interesting that placental macrophages have some characteristics in common with the rapidly-adherent cells classified as placental by Gosden and Brock (1978a). Fox (1978) has described placental macrophages as ovoid cells with an eccentrically placed nucleus and a cytoplasm which was usually, though not invariably, coarsely vacuolated. Placental cells, according to Gosden and Brock (1978a), are ovoid cells with an eccentrically placed nucleus appearing as a cap at one end of the cell, but with no cytoplasmic granulations, inclusions or vacuoles.

The description of syncytiotrophoblastic cells of placenta (Fox, 1978) is also similar to that of "fetal distress cells" (Gosden and Brock, 1978b). Syncytiotrophoblastic cells have a cytoplasm which is commonly vacuolated, while some of the vacuoles contain lipid. Under electron microscopy the endoplasmic reticulum appears to communicate with the perinuclear spaces which are often dilated to form juxtanuclear vacuoles (Fox, 1978). The juxtanuclear

vacuoles of syncytiotrophoblastic cells are moderately dilated in many areas of molar trophoblast and there are large vacuoles of the "lagoon type". This is probably due to the accumulation of transport fluid and an impaired excretory function of the syncytiotrophoblastic cells (Fox and Khargonkor, 1971). Fetal distress cells have been described as having the characteristic feature of large irregular vacuoles in the cytoplasm, usually placed immediately adjacent to the nuclear membrane (Gosden and Brock, 1978b).

Fox (1978) observed that 20-40% of placentae where there has been a spontaneous abortion have hydropic and/or hydatiform hyperplasia of the villi. In placentae of Rhesus isoimmunization, pre-eclampsia, intrauterine hypoxia and low birthweight, there is an increase in cytotrophoblastic cells.

It was concluded that the "fetal distress" cells described by Gosden and Brock (1978b) could be either macrophages or trophoblastic cells. Fox and Khargonkor (1970) found, in their study of intact term placental villi and cultures derived from them, that only placental macrophages were positive for NSAE stain. In contrast, Wood et al. (1978), in their studies of placentae from 4 weeks of gestation to full term, found that placental macrophages were NSAE positive, but that a "significant" proportion of trophoblastic cells were also positive. In this study it was hoped that the use of NSAE stain would detect placental macrophages and trophoblastic cells in sufficient numbers to identify potential complications during pregnancy. NSAE stained macrophages positively, but did not seem to be the ideal method for identifying trophoblastic cells. In long-term cultures of AF cells (considered trophoblastic in origin), only 2% of the cells were positive for NSAE stain.

This study has proved that the NSAE positive cells of amniotic fluids are short-lived and diminish considerably in number when cultured (Table 30). This could be explained by the absence in culture of a colony stimulating factor required for the proliferation and differentiation of mononuclear phagocytic cells (Tushinsky et al., 1982). Therefore, cytospin preparations are considered as the best method for studying these cells. Further studies should be carried out in larger samples, but trophoblastic cells should be identified by methods other than their positive staining reaction to NSAE. The presence of hCG might be a good marker for trophoblastic cells.

In cell suspensions from 12 to 16 week gestation human placental trophoblast, Wood et al. (1978b) found that 90% of the cells were positive for Fc receptors, and of these cells 95% were either NSAE positive or phagocytic. Phagocytic studies of rapidly-adherent AFC (Medina-Gómez, 1979) showed that the only pregnancy which ended in a low birthweight infant (Tables 32 and 33), and had no fetal distress cells in the amniotic fluid, also had a very small placenta (200 g). According to Fox (1978), the normal placental weight ranges from 430 to 650 g, but it is related to birthweight and in his consensus is not an accurate or valuable measure. Whether the amniotic fluid cell content is related to the placental weight and size is not yet known. It would be very interesting to undertake a prospective study using ultrasound to measure the size of the placenta (Adamson, 1978) and compare it to the outcome. This could be of great importance in the detection of high risk pregnancy and the eventual delivery of a low birthweight infant which is associated with second trimester elevated maternal serum AFP (Brock, 1980).

TABLE 32 NORMAL AND NTD AMNIOTIC FLUIDS CLASSIFIED ACCORDING TO TYPE OF FLUID, INDICATION FOR AMNIOCENTESIS AND PHAGOCYTIC PROPERTIES (from Medina-Gómez, 1979)

No.	Classification	Gestation in weeks	Reason for amniocentesis	Amniotic AFP g/ml	Phagocytic Index	Degree of Phagocytosis	Total No. of cells	% Adherence	Outcome and complications of pregnancy
NORMAL AMNIOTIC FLUIDS									
931	A/b	22(S)	1	0.9	27%	+ +	11	1	
950	A/b	18(S)	1	1.0	0%		0	0	Threatened abortion: 8 & 11/52
956	A/B	19(S)	1	3.1	39%	+	18	1	LBW (2.1 kg)
971	a/B	21(S)	1	3.1	11%	+	9	1	
974	a/b	18(S)	1	3.1	0%		12	1	
NTD AMNIOTIC FLUIDS									
939	a/B	19-20	1	78.0	83%	+ + +	725	37	Anencephaly
951	a/B	18	1	38.8	87%	+	187	18	Acranium + Encephalocele

Classification:
a Total cell count < 70,000
A Total cell count > 70,000
b < 100,000 RBC
B > 100,000 RBC
(S) Gestational age given by scan

Reason for amniocentesis:
1 - High maternal serum AFP
2 - Previous NTD
3 - Two miscarriages
4 - Three miscarriages

TABLE 33 FETAL DISTRESS AMNIOTIC FLUIDS CLASSIFIED ACCORDING TO TYPE OF FLUID, INDICATION FOR AMNIOCENTESIS AND PHAGOCYTIC PROPERTIES (from Medina-Gómez, 1979)

No.	Classification	Gestation in weeks	Reason for amniocentesis	Amniotic AFP g/ml	Phagocytic Index	Degree of Phagocytosis	Total No. of cells	% Adherence	Outcome and complications of pregnancy
1% FETAL DISTRESS CELLS									
936	A/B	19	1	3.9	29%	+ +	254	12	
938	A/b	21	1	3.9	22%	+	100	6	Labile hypertension
952	A/b	19	2	3.6	33%	+	15	1	
954	a/b	17	1	2.6	0%		13	2	Uterine contractions 34/52
955	a/b	20(S)	4	1.6	30%	+ +	30	2	
959	A/b	18(S)	3	3.2	0%		93	3	
960	A/b	19(S)	1	3.2	9%	+ +	78	3	
961	a/B	19(S)	1	4.8	5%	+ + +	24	2	Oedema 35/52
972	A/b	20(S)	1	2.3	80%	+ + +	15	1	
1-2% FETAL DISTRESS CELLS									
935	a/B	19	2	3.0	25%	+ +	55	6	
937	A/b	22	1	1.9	59%	+ +	44	3	
962	A/b	18	1	2.1	26%	+	110	3	LBW(2.3 kg)
969	A/B	18	1	2.5	33%	+ +	105	5	LBW(2.16 kg) Fulminating eclampsia
3% OR MORE FETAL DISTRESS CELLS									
934	a/b	19	1	3.9	84%	+ + +	114	7	Uterine contractions 25/52
968	A/B	18	1	3.3	96%	+ +	99	6	Oedema 28/52
975	A/b	19	1	2.4	80%	+ +	81	8	

LBW - low birthweight

See footnote Table 32.

2. Significance in antenatal diagnosis

The significance of placental macrophages is not fully understood, but materno-fetal transport of IgG has been suggested (Börner et al., 1973; Johnson and Faulk, 1978; van der Meulen et al., 1980). Wood et al. (1978b) found that IgG adsorption to the trophoblast was limited to a few villi in term placentae, while 50 to 90% were positive in younger placentae. The majority of IgG was associated with Ig Fc receptors of stromal macrophages, suggesting the following functions for placental macrophages (Wood et al., 1978b):

i) to serve as a secondary line of defence against invasion of the fetus by infectious agents.

ii) the removal of antifetal antibodies from maternal IgG.

iii) the suppression of immune response to fetal transplantation antigens.

Contractor and Krakaner (1976) have added a fourth role:

iv) as a nutritive mechanism of the fetus or the placenta.

These functions might explain the relationship between placental macrophages and high risk pregnancy, spontaneous abortion, pre-eclampsia and delivery of low birthweight infants.

The presence of macrophages in amniotic fluids from fetuses with a NTD is more difficult to explain. Whether these macrophages are a response only to tissue damage, or whether there has been a previous antigenic or immunologic stimulus is not known. Bell et al. (1981), in their findings of meninges from fetuses with anencephaly, suggested that disintegration of well-formed neural tissue does

occur, and that excessive vascularity may not be accounted for totally as an inflammatory reaction.

B. Neural Cells in Amniotic Fluids

1. Origin

Neural cells were observed in uncultured and both short- and long-term cultures of amniotic fluids from fetuses with a NTD.

The obvious source of the neural cells is the open neural lesion in encephalocele, spina bifida or the area cerebrovasculosa and spinal meninges in anencephaly (Bell et al., 1981). The open lesion could yield neural cells such as neuroblasts, glial cells and neurons, as well as other non-neural cell types such as macrophages, meningeal cells, fibroblasts and endothelial cells. A varying number of these cell types would slough off into the amniotic fluid depending on the site and severity of the lesion.

a) Embryopathology

There are two main theories for explaining the occurrence of NTD: 1) failure of closure of the neural tube (Marin-Padilla, 1970); 2) primary neural tube closure with reopening (Gardner, 1973).

The neural tube closure starts at around the 25th day of gestation with the cranial neuropore, followed by closure of the caudal neuropore two days thereafter. After neural tube closure, the neuroepithelial cells, which form a pseudostratified epithelium, divide and start differentiating. Neuroblasts will yield neurons,

gliablasts will give rise to astrocytes and oligodendrocytes and neuroepithelial cells will also differentiate into ependymal cells (Langman, 1981) (Fig. 16). The development of the embryo will therefore take place at two levels: the macroscopical level where the neural tube will give rise to brain and spinal cord and the microscopical level where neuroepithelial cells will differentiate into neurons, glial cells and ependymal cells.

The neural tube defects are classified according to the severity of the lesion (Fig. 17) into:

i) Anencephaly: The brain is represented by a mass of degenerated tissue exposed to the surface.

ii) Spina bifida:

1) occulta: localized failure of dorsal portions of the vertebra to fuse.

2) meningocele: more than one or two vertebrae are involved and the meninges of the spinal cord bulge through the opening sac covered with skin.

3) meningomyelocele: the sac, covered by a thin easily torn membrane, contains not only meninges but spinal cord and its nerves.

iii) Rachischisis: Failure of the neural groove to close so the nervous tissue is widely exposed on the surface.

b) Use of AFP in the antenatal diagnosis of NTD

Screening for fetal NTD by measurement of maternal serum AFP (Report of U.K. Collaborative Study on AFP in Relation to Neural

FIG. 16: Schematic diagram showing the origin of the nerve cell and the various types of glial cells. The neuroblast, the fibrillar and protoplasmic astrocytes and the ependymal cells originate from the neuroepithelial cells. The microglia develop from mesenchymal cells. The origin of the oligodendroglia remains in doubt.

(from Langman, 1981).

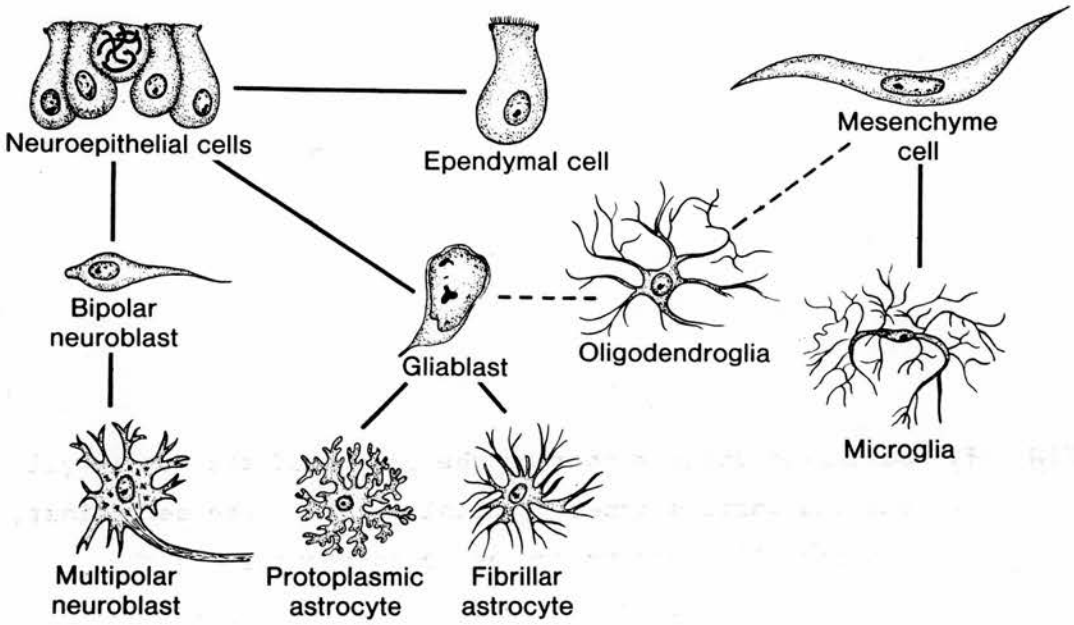
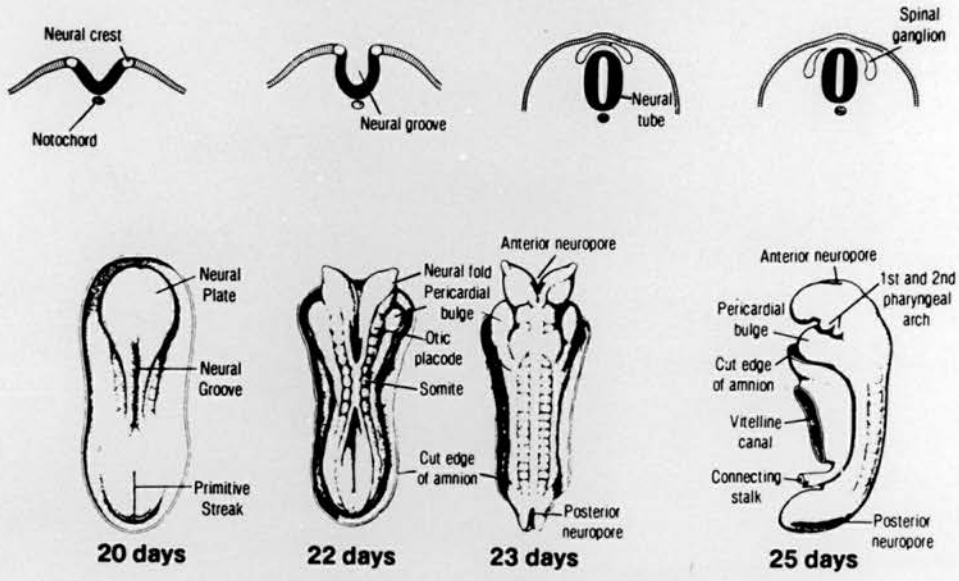
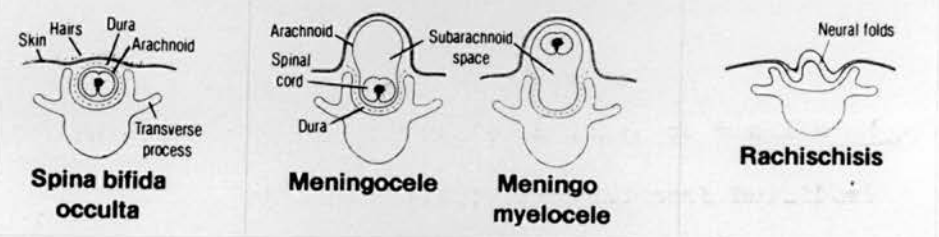

Neuroepithelial cells
Ependymal cell
Mesenchyme cell
Bipolar neuroblast
Gliablast
Oligodendroglia
Microglia
Multipolar neuroblast
Protoplasmic astrocyte
Fibrillar astrocyte

FIG. 17: Normal and abnormal development of the neural tube (modified from Langman, 1981)

Normal Development Neural Tube



Abnormal Development of Neural Tube



Anencephaly

Tube Defects, 1977) can detect 88% of anencephaly and 79% of open spina bifida (using 2x the median). However, raised maternal serum AFP is not specific for NTD, but can also be an indicator of other physiological and pathological states such as multiple pregnancy, threatened abortion, intrauterine death and low birthweight (Brock, 1980).

This is why this screening technique has to be accompanied by a more satisfactory diagnostic test such as amniotic fluid AFP determination. Before amniocentesis is performed, causes of raised maternal serum AFP should be investigated with clinical and ultrasound examinations. This will exclude some pregnancies where gestational dates have been underestimated, and cases of twins and intrauterine death or threatened abortion. It has also been suggested that a second blood sample would diminish the false positives by a third (Report of U.K. Collaborative Study on AFP in Relation to Neural Tube Defects, 1977). The sensitivity of the amniotic fluid AFP test is 98% for both anencephaly and open spina bifida (U.K. Collaborative Study, 1979). The specificity, taking a cut off point of three standard deviations above the mean, is about 99.5%. Raised amniotic AFP can also be caused by fetal blood contamination, fetal death and other non-neurological congenital abnormalities such as congenital nephrosis, exomphalos, esophageal atresia, sacrococcygeal teratoma and Meckel's syndrome (Seppälä, 1977).

c) Neural cells in culture

Most studies of neural cells in culture have been carried out using rodent or chick embryo cells. Recently, the time of

appearance of neural cells *in vivo* and in dissociated cell cultures of embryonic rat brain has been studied (Tables 34 and 35). In order to consider the relevance of these findings to human fetal development, one has to make a comparison between the neural development of rat and man (Table 36).

In the present study, cinemicrography showed the presence of undifferentiated cells (neuroblasts) through their different stages of differentiation into neurons and glial cells with characteristics of astrocytes and microglia. The differentiation of the neural cells in cultures of amniotic fluids from fetuses with a NTD was influenced by the initial neural cell density, cell-cell interactions, time of culture and gestational age of the fetus.

More cells with neural characteristics will attach and differentiate in amniotic fluids from fetuses with anencephaly than in amniotic fluids from fetuses with spina bifida or encephalocele.

Cell-cell interaction has been described previously as an important factor in cell differentiation (Choi and Lapham, 1976; Foucaud et al., 1982). In the present study, neurons were only observed in long-term AFC cultures of fetuses with a NTD and were always in close association with G-E cells which often acted as substratum for the neurons. Problems in culturing dissociated neurons are due to the inability of neurons to adhere to the culture surface, which resulted in their being washed out when the first medium change was carried out (Raju *et al.*, 1981). To increase the adherence of dissociated neurons, culture surfaces have been coated with a variety of artificial substrates (collagen, polylysine) and

TABLE 34: TIMES OF APPEARANCE OF NEURAL CELLS *IN VIVO* AND IN DISSOCIATED CELL CULTURES OF EMBRYONIC RAT BRAIN (from Abney *et al.*, 1981; Raju *et al.*, 1981)

	Marker	*In vivo* (days)	*In vivo* (days)		
			10	13	
Macrophages	Fc receptor	≤ 11	≤ 1	≤ 1	Days *in vitro*
			Varied greatly in size and shape		Morphology *in vitro*
Astrocyte precursors	Ran 2+/GFAP-	≤ 11	≤ 1	≤ 1	Days *in vitro*
			Clusters, often beneath neurons, smaller and darker than fibroblasts		Morphology *in vitro*
Astrocytes	GFAP+	15-16 (brain) 18 (spinal cord)	5-6	3	Days *in vitro*
			Flat cells, process bearing cells (attached to glass) did *not* form inter-connecting network of fibres as neurons; few star-shaped astrocytes		Morphology *in vitro*
Ependymal cells	Beating cilia	17-18	7-8	4-5	Days *in vitro*
			Tightly packed clusters		Morphology *in vitro*
Oligodendrocytes	Galacto cerebroside	2-3 after birth	13-14	10-11	Days *in vitro*
			Cells tended to line up among bundles of neurites & extend processes around neuronal cell bodies		Morphology *in vitro*

TABLE 35 TIMES OF APPEARANCE OF NEURONS IN VIVO AND DISSOCIATED CELL CULTURES OF EMBRYONIC RAT BRAIN

(from Sotelo et al., 1980; Abney et al., 1981; Raju et al., 1981)

Marker	In vivo (days)	In vivo (days)							
	11	8-10	10	11	12	13	14	≥ 16	
Tetanus toxin	≤ 11		≤ 1			≤ 1			Days in vitro
Neurofilaments	12	-		1 (10%)	2	5-6	1-2		
		Clumps of process bearing cells		Dispersed monopolar + bipolar cells	Unipolar and bipolar cells, clusters of 3-4 cell bodies	Dispersed large neurons with long processes	Small clusters Islets Multi-polar neurons	Decrease in neuronal yield	Morphology in vitro
Silver staining				28					Days in vitro
				Large clusters. Neurites from several neurons form thick straight bundles which after some distance diverge in different directions					Morphology in vitro

TABLE 36 BRAIN GROWTH AND DIFFERENTIATION IN RAT AND MAN FOLLOWING NEURAL TUBE FORMATION *

Phase of Growth	Developmental Age	
	Rat	Man
Neuroblast multiplication	Last 1/3 pregnancy	10 - 18 weeks gestation
Rapid brain growth	Birth - 21 days	18 weeks gestation - 4th year
Spongioblast multiplication and dendritic arborization	0 - 9 days	18 weeks gestation - 2nd year
Initiation of astrogliogenesis		7th week gestation **
Initiation of myelination		16 weeks gestation ***
Myelin synthesis and functional synaptic connection	10 - 21 days	32 weeks gestation - 4th year

* Modified from Dobbing (1974)

** Choi and Lapham (1978, 1980)

*** Langman (1981)

cell-derived substrates (substratum of killed non-neuronal cells; extracellular substrate-associated material apparently produced by non-neuronal cells) (Raju et al., 1981; Hawrot, 1980). In this study differentiating neurons, either from the encephaloceles or amniotic fluids from fetuses with NTD, adhered poorly to glass, showing instead increased cell-cell adherence (Rutishauser et al., 1982). Differentiating neurons attached firmly to G-E cells and moved with them. This type of neuronal-glial adhesion and migration might be important in the concept of guidance of migrating neurons by radial glia (Rakic, 1975).

2. Significance in antenatal diagnosis

The presence of different neural cell types and their stage of differentiation could have important implications in antenatal diagnosis of NTD. Recently, studies on immunocytochemistry of AFC from fetuses with NTD (anencephaly or encephalocele) have shown them to be positive for glial proteins, protein S-100 (Sarkar et al., 1980), glial fibrillary acidic protein (GFAP) (Aula et al., 1980; Cremer et al., 1981a; von Koskull et al., 1981), and neuronal neurofilaments (von Koskull et al., 1981).

GFAP positive cells in amniotic fluids from fetuses with anencephaly are considered to be astrocytes (Aula et al.,1980; Cremer et al., 1981a; von Koskull et al., 1981). GFAP positive cells were mainly spread evenly over the coverslips and grew in monolayers both in primary and secondary cultures (von Koskull et al., 1981). In primary cultures, cell conglomerates contained many GFAP positive cells, and morphologically the cells showed extensive

variation (Aula et al., 1980; Cremer et al., 1981a; von Koskull et al., 1981). The morphological variation of primary cultures of GFAP positive cells could indicate a different stage of differentiation of glial cells (Lim, 1980). The present cinemicrographic study showed that the tug-of-war movement, characteristic of differentiating or differentiated astrocytes (Lim, 1976; Lim, 1980), was more evident in short-term cultures than in long-term cultures. This could be explained by the fact that a glial maturation factor seems to be required to maintain the differentiated properties of astrocytes in culture (Lim, 1980). GFAP, however, is present in both undifferentiated glioblasts and differentiated glial cells (Bignami et al., 1980; Lim, 1980).

The apparent discordance in the number of GFAP positive cells present in amniotic fluids from fetuses with anencephaly or encephalocele (Cremer et al., 1981a; von Koskull et al., 1981) could be due to the gestational age of the fetus and the duration in culture of the AFC. The appearance of immunohistological markers of glial and neuronal cells in vitro follow the same time sequence which would occur in vivo (Abney et al., 1981; Raju et al., 1981). The different markers will appear at various times either in vivo or in vitro with their concentration changing through culture (Abney et al., 1981; Bock et al., 1980; Raju et al., 1981). Furthermore, in vivo GFAP appears in embryonic rat brain at 15 to 16 days of gestation, but not until 18 days of gestation in spinal cord (Abney et al., 1981; Raju et al., 1981). In the human fetus astrogliogenesis starts as early as the 7th week of gestation (Choi and Lapham, 1978, 1980). GFAP is not only present in astrocytes

but also in ependymal cells of human fetuses between the 15th week of gestation and term, while both astrocytes and ependymal cells are increased in cases of CNS damage (Roessmann et al., 1980). GFAP has been found in cultured astrocytes from human fetuses as early as 12 weeks of gestation.

Cremer et al. (1981a) found the highest proportion (80%) of AFC positive for GFAP in a case of encephalocele. This result raises the question of whether the encephalocele was punctured during amniocentesis (which has been reported before; Stirrat et al., 1979), or whether the later gestational age of 30 weeks has any significance in detecting more GFAP positive cells.

It therefore seems that the number of neural cells (neuronal or glial) in amniotic fluids from fetuses with a NTD will depend on the location of the defect (brain or spinal cord), the extent of the lesion and the gestational age of the fetus. If these cells are cultured, they will be subjected to selection by the effects of tissue culture and cell-cell interaction. The cells will differentiate in vitro as they would have differentiated in vivo and the immunohistological markers will be modified according to their differentiation and time in culture.

It is important to be cautious about the specificity of GFAP in the detection of NTD. GFAP is positive in glial cells of the enteric nervous system of rats (Jessen and Mirsky, 1980). Cremer et al. (1981a) mention one case of fetal omphalocele where the AFC were negative for GFAP. However, these amniotic fluids can have an acetylcholinesterase band and it has been suggested that the

enzyme derives from peripheral nervous tissue such as intestinal nerve plexi (Wald et al., 1980). Thus, caution in diagnosis should be shown until it is established whether some GFAP positive cells of the enteric nervous system of a fetus with omphalocele or gastroschisis could slough off into the amniotic fluid.

C. Other Amniotic Fluid Cell Types

1. Origin

This study shows that primary cultures from normal and abnormal amniotic fluids consist of a very heterogeneous population of cells. Both normal and abnormal amniotic fluids contain the E, F and AF cell types described by Hoehn et al. (1974). The origin of AFC has been greatly debated and many classifications have been made. Recently, Priest et al. (1979) have suggested trophoblast as the origin of AF cells and placental stroma as the origin of F cells. Hoehn et al. (1975) suggested fetal urine as the origin of E cells.

In the present study, cinemicrography and NSAE stain confirmed the classification of F cells as fibroblastoid, E cells as epithelioid and AF cells as trophoblastic.

The E cell type displayed a distinct behaviour, moving as a coherent pavement-like sheet of cells. In contrast, AF cells displayed either cytotrophoblastic or syncytiotrophoblastic characteristics. They formed cell clusters which had a type of

locomotion that was intermediate between single cells and extended sheets, or they moved like syncytial masses.

Trophoblastic cells are said to differentiate *in vitro* from cytotrophoblast to syncytiotrophoblast (Lueck and Aladjem, 1980; Aladjem and Lueck, 1981). If AF cells differentiate in the same way as trophoblastic cells *in vitro*, this would explain the results obtained by Virtanen *et al*. (1981b) and Cremer *et al*. (1981b) using immunofluorescence to detect intermediate filaments in AFC cultures. Their studies showed that AF cells are positive both for keratin and vimentin. Keratin filaments are considered characteristic of cells of epithelioid origin and vimentin of cells of mesenchymal origin (Lazarides *et al*., 1980). However, vimentin filaments can be expressed during adaptation in culture. Both Virtanen *et al*. (1981b) and Cremer *et al*. (1981b) found cell heterogeneity within AF cell colonies. Virtanen *et al*. (1981b) classified AFC according to their morphology and keratin pattern into: (a) pleomorphic cell types E-1 (previous AF cells), E-2 and E-3; (b) multinucleated cell type E-5; and (c) E-4 which consisted of small dense colonies. The E-4 cell type would correspond to the cells which moved like a coherent sheet of cells (E type of Hoehn *et al*., 1974) and their origin could be fetal urine as suggested by Hoehn *et al*. (1975). E-1, E-2, E-3 and E-5 seem to fit with the description of trophoblastic cells in culture, being either cytotrophoblast at different stages of differentiation (E-1, E-2 and E-3) or syncytiotrophoblast (E-5). AF cells and trophoblast are characterized by the production of human chorionic gonadotrophin (hCG) (Laundon *et al*., 1981). It would be interesting to perform a double immunofluorescent study to

detect the presence of both intermediate filaments and hCG in the same amniotic fluid cell type in order to clarify their origin.

2. Significance in antenatal diagnosis

AF cells are the main component of amniotic fluid cultures (Hoehn et al., 1974). Therefore a trophoblastic origin would explain why AFC are so difficult to culture and karyotype. As trophoblastic cells, AF cells would require several days to attach to the substrate (Lueck and Aladjem, 1980) and then several days in culture before they started dividing (Lueck and Aladjem, 1980; Valenti, 1965). The stage of cell differentiation of trophoblastic cells would depend as in neural cells (section V, B.2) on the gestational age of the pregnancy (Lueck and Aladjem, 1980; Valenti, 1965).

The study of trophoblastic cells in culture has been hampered by the difficulties involved in their culture, and most studies have been performed on trophoblast at 38 to 42 weeks of gestation (Aladjem and Lueck, 1981; Lueck and Aladjem, 1980). The availability of AFC cultures should offer the opportunity of studying trophoblastic cells at an earlier gestation.

VI. CONCLUSIONS

Normal amniotic fluids contain mainly squamous cells, a few macrophages and a very few "colony forming cells" (CFC). The CFC will give rise to E, F and AF cell types observed in long-term amniotic fluid cultures (Hoehn *et al.*, 1974). Cinemicrographic and NSAE results confirmed the classification of E cells as epithelioid, F cells as fibroblastoid and AF cells as trophoblastic. Although amniotic fluid cultures are said to grow in monolayers (Martin, 1980), it was found that primary cultures kept *in vitro* for as long as 60 days would become multilayered. F cells formed the orthogonal layers described by Elsdale and Bard (1972), E cells formed dermatoglyph patterns as described by Green and Thomas (1978), and the mixture of F and E cells formed a similar organization to that described by Bard (1979) in human embryonic kidney.

AF cells are the main cell type in long-term cultures of amniotic fluids (Hoehn *et al.*, 1974). Cinemicrography and NSAE pointed towards a trophoblastic origin as has been suggested by Priest *et al.* (1979). The heterogeneity of AF cells can be explained as the effect of differentiation in culture of cyto-trophoblast into syncytiotrophoblast. The trophoblastic origin of AF cells would explain why amniotic fluids are so difficult to culture and karyotype.

Macrophages are present in both normal and abnormal amniotic fluids, with the highest number of macrophages being observed in amniotic fluids from fetuses with anencephaly. Macrophages, however, are short-lived cells and almost non-existent

in long-term cultures of amniotic fluids. The origin of macrophages in normal amniotic fluids is most probably placenta and in abnormal amniotic fluids the fetal lesion, lung and/or placenta. In amniotic fluids from fetuses with a NTD the macrophages not only come from the surface of the fetal lesion, such as area cerebrovasculosa in fetuses with anencephaly (Papp and Bell, 1979; Chapman et al., 1981), but were also present in the spinal cord from a fetus with anencephaly and the CSF from a fetus with spina bifida. It is not known whether the high number of macrophages in amniotic fluids of fetuses with a NTD is a response to the presence of the lesion alone or to a previous antigenic or immunologic stimulus.

Neural cells in amniotic fluids from fetuses with a NTD are extremely variable in number and they differentiate in culture. Cinemicrography suggested that these neural cells consisted of neuroblasts which differentiated into neurons, astrocytes which seem to dedifferentiate into glioblasts, and microglia. Differentiation of neural cells is influenced by the initial neural cell density, cell-cell interactions, time of culture and gestational age of the fetus. The quantification of neural cells (neuronal or glial) present in amniotic fluids from fetuses with a NTD, using immunohistochemistry, will depend on the location of the defect (brain or spinal cord), extent of the lesion and gestational age of the fetus. When these cells are cultured, they are subjected to selection by the effects of tissue culture and cell-cell interaction. The cells will differentiate in vitro following the same sequence as in vivo and the immunohistological markers will be modified according to their differentiation and time in culture.

Few workers have studied uncultured amniotic fluids. This study confirmed that the use of cytospin preparations is a simple, useful technique which requires no complicated or expensive cell culture and will give permanent preparations. Cytospin preparations will also give a better idea of the initial content of amniotic fluids. Although the use of NSAE stain failed to detect complications of pregnancy, such as missed abortion and severe pre-eclampsia, larger studies should be undertaken using not only a marker for macrophages but also for trophoblastic cells. Cytospin preparations are useful in antenatal diagnosis of fetal malformation, using cell morphology and histochemistry. Markers for undifferentiated neural cells (neuroblasts) could give more significant results than the use of markers for differentiated neural cells. Amniotic fluids from fetuses with anencephaly are a good source of neural cells at different stages of gestation. These cells could be used advantageously in developmental studies of human fetal neural cells in culture.

Further studies of amniotic fluids and fetal cells in congenital malformations and pathological pregnancies should be complemented by extensive pathological studies of the malformed fetuses and their placentae. This might shed some light on their aetiology and help us understand the feto-maternal relationship.

BIBLIOGRAPHY

Abney, E.R., Bartlett, P.P. and Raff, M.C. (1981) Astrocytes, ependymal cells, and oligodendrocytes develop on schedule in dissociated cell cultures of embryonic rat brain. Developmental Biology; 83: 301-310.

Adamson, K. (1978) Fetal growth: obstetric implications. In: Falkner, F. and Tanner, J.H. Eds. Human Growth. 1. Principles and Prenatal Growth. pp. 609-619. Baillière Tindall, London.

Aladjem, S. and Lueck, J. (1981) Morphologic characteristics of the normal term human trophoblast maintained in prolonged in vitro cultures. British Journal of Obstetrics and Gynaecology; 88: 287-293.

Albrecht-Buehler, G. (1977) The phagokinetic tracks of 3T3 cells. Cell; 11: 395-404.

Alter, B.P. (1981) Prenatal diagnosis of haemoglobinopathies: a status report. Lancet; II; 1152-1155.

Antanitus, D.S., Choi, B.H. and Lapham, L.W. (1975) Immunofluorescence staining of astrocytes in vitro using antiserum to glial fibrillary acidic protein. Brain Research; 89: 363-367.

Aula, P., von Koskull, H., Teramo, K., Karjalainen, O., Virtanen, I., Lehto, V.P. and Dahl, D. (1980) Glial origin of rapidly adhering amniotic fluid cells. British Medical Journal; 281: 1456-1457.

Bard, J.B.L. (1979) Epithelial fibroblastic organization in cultures grown from human embryonic kidney: its significance for morphogenesis in vivo. Journal of Cell Science; 39: 291-298.

Bell, J.E., Gordon, A. and Maloney, A.F.J. (1981) Abnormalities of the spinal meninges in anencephalic fetuses. Journal of Pathology; 133: 131-144.

Bergström, S. (1979) Amniotic fluid cell exfoliation in early human pregnancy. Acta Obstetricia et Gynecologica Scandinavica; 58: 353-360.

Birdwell, C.R., Gospodarowicz, D. and Nicolson, G.L. (1978) Identification, localization and role of fibronectin in cultured bovine endothelial cells. Proceedings of the National Academy of Sciences of the United States of America; 75: 3273-3277.

Bignami, A., Dahl, D. and Rueger, D.C. (1980) Glial fibrillary acidic protein (GFA) in normal neural cells and in pathological conditions. Advances in Cellular Neurobiology; 1: 285-310.

Bock, E., Yavin, Z., Jørgensen, O.S. and Yavin, E. (1980) Nervous system - specific proteins in developing rat cerebral cells in culture. Journal of Neurochemistry; 35: 1297-1302.

Booher, J., Sensenbrenner, M. and Mandel, P. (1973) Neuroblastoma cell differentiation: a tissue culture study using time-lapse cinematography. Neurobiology; 3: 335-338.

Bőrner, P.H., Deicher, H., Heide, K. and Reinecke, J. (1973) Evidence for the participation of the Fc portion of immunoglobulin G in maternofoetal immunoglobulin transfer in the human. International Congress of Immunology in Obstetrics and Gynecology. pp. 272-274.

Bowser-Riley, S. (1978) Current problems of amniotic fluid cell culture. In: Scrimgeour, J.B. Ed. Towards the Prevention of Fetal Malformation. pp. 157-164. Edinburgh University Press.

Bray, D. and Bartlett Bunge, M. (1973) The growth cone in neurite extension. In: Locomotion of Tissue Cells, Ciba Foundation Symposium 14 (New Series) pp. 195-209. Elsevier Scientific Publishing Company, Amsterdam.

Brock, D.J.H. (1978) Inborn errors of metabolism. In: Brock, D.J.H. and Mayo, O. Eds. The Biochemical Genetics of Man. pp. 470-544. Academic Press, London, New York, San Francisco.

Brock, D.J.H. (1980) Feto-specific proteins in pre-natal diagnosis. Molecular Aspects of Medicine; 3: 431-554.

Brock, D.J.H. (1981) The use of amniotic fluid AFP action limits in diagnosing open neural tube defects. Prenatal Diagnosis; 1: 11-16.

Capo, C., Bongrand, P., Benoliel, A.M. and Depieds, R. (1974) Phagocytosis. Journal of Theoretical Biology; 47: 177-188.

Casadei, R., D'Ablaing, G., Kaplan, B.J. and Schwinn, C.P. (1973) A cytologic study of amniotic fluid. Acta Cytologica; 17: 289-298.

Chapman, P.A., Blenkinsopp, W.K. and Lewin, B.V. (1981) The detection of neural tube closure defects by exfoliative cytology of amniotic fluid. Acta Cytologica; 25: 367-372.

Chen, L.B., Maitland, N., Gallimore, P.H., McDougall, J.K. (1977) Detection of large external transformation-sensitive protein on some epithelial cells. Experimental Cell Research; 106: 39-46.

Choi, B.H. and Lapham, L.W. (1976) Interaction of neurons and astrocytes during growth and development of human fetal brain in vitro. Experimental Molecular Pathology; 24: 110-125.

Choi, B.H. and Lapham, L.W. (1978) Radial glia in the human fetal cerebrum: a combined golgi, immunofluorescent and electron microscopic study. Brain Research; 148: 295-311.

Choi, B.H. and Lapham, L.W. (1980) Evolution of Bergmann glia in developing human fetal cerebellum: a golgi, electron microscopic and immunofluorescent study. Brain Research; 190: 369-383.

Cohen, I. (1971) Origine tisulaire des cellules du liquide amniotique. La Presse Medicale; 79 2539-2540.

Colombani, P.M. and Cunningham, M.D. (1977) Perinatal aspects of omphalocele and gastroschisis. American Journal of Diseases in Children; 131: 1386-1388.

Contractor, S.F. and Krakaner, K. (1976) Pinocytosis and intracellular digestion of ^{125}I-labelled haemoglobin by trophoblastic cells in tissue culture in the presence and absence of serum. Journal of Cell Science; 21: 595-607.

Cremer, M., Schachner, M., Cremer, T., Schmidt, W. and Voigtländer, T. (1981 a) Demonstration of astrocytes in cultured amniotic fluid cells of three cases with neural tube defect. Human Genetics; 56: 365-370.

Cremer, M., Treiss, I., Cremer, T., Hager, D. and Franke, W.W. (1981 b) Characterization of cells of amniotic fluids by immunological identification of intermediate-sized filaments: presence of cells of different tissue origin. Human Genetics; 59: 373-379.

Crouch, E., Balian, G., Holbrook, K., Duksin, D. and Bornstein, P. (1978) Amniotic fluid fibronectin. Characterization and synthesis by cells in culture. Journal of Cell Biology; 78: 701-715.

Crouch, E. and Bornstein, P. (1978) Collagen synthesis by human amniotic fluid cells in culture: characterization of a procollagen with three identical pro α 1 (I) chains. Biochemistry; 17: 5499-5509.

Crouch, E. and Bornstein, P. (1979) Characterization of a type IV procollagen synthesized by human amniotic fluid cells in culture. Journal of Biological Chemistry; 254: 4197-4204.

Cutz, E. and Conen, P.E. (1978) Macrophage and epithelial cells in human amniotic fluid: transmission and scanning electron microscopic study. American Journal of Anatomy; 151: 87-102.

De Vitry, F., Picart, R., Jacque, C., Legault, L., Dupovey, P. and Tixier-Vidal, A. (1980) Presumptive common precursor for neuronal and glial cell lineages in mouse hypothalamus. Proceedings of the National Academy of Sciences of the United States of America; 77: 4165-4169.

Dobbing, J. (1974) The later development of the brain and its vulnerability. In: Davis, J.A. and Dobbing, J. Eds. Scientific Foundation of Paediatrics pp. 565-577. W.B. Saunders, Philadelphia.

Elsdale, T. and Bard, J. (1972) Cellular interactions in mass cultures of human diploid fibroblasts. Nature; 236: 152-155.

Elsdale, T. and Bard, J. (1974) Cellular interactions in morphogenesis of epithelial mesenchymal systems. Journal of Cell Biology; 63: 343-349.

Emery, J.L. (1974) Evidence of antigenic active substances in the amniotic fluid in children with open neurospinal dysraphism. Developmental Medicine and Child Neurology; 16 Supp. 32: 154.

Faulk, W.P. (1980) Immunology of the materno foetal relationship. In: Fougereau, M. and Dausset, J. Eds. Fourth International Congress of Immunology. pp. 1094-1117. Academic Press, London, New York, San Francisco.

Fields, K.L. (1979) Cell type specific antigens of cells of the central and peripheral nervous system. Current Topics in Developmental Biology; 13: 237-257.

Foucaud, B., Reeb, R., Sensenbrenner, M. and Gombos, G. (1982) Kinetic and morphological analysis of the preferential adhesion of chick embryo neuronal cells to astroglial cells in culture. Experimental Cell Research; 137: 285-294.

Fox, H. (1978) Pathology of the placenta. Major Problems in Pathology. VII. W.B. Saunders Company Ltd., London, Philadelphia, Toronto.

Fox, H. and Kharkongor, F.N. (1970) Morphology and enzyme histochemistry of cells derived from placental villi tissue culture. Journal of Pathology; 101: 267-276.

Fox, H. and Kharkongor, N.F. (1971) The ultrastructure of molar trophoblast. Journal of Obstetrics and Gynaecology British Commonwealth; 78: 652-659.

Gail, M. (1973) Time-lapse studies on the motility of fibroblasts in tissue culture. In: Locomotion of Tissue Cells. Ciba Foundation Symposium. 14 (New Series). pp. 287-310. Elsevier Scientific Publishing Company, Amsterdam.

Galjaard, H. (1980) Quantitative cytochemical analysis of (single) cultured cells. In: Trends in Enzyme Histochemistry and Cytochemistry. Ciba Foundation Symposium 73. pp. 161-180. Excerpta Medica, Amsterdam, Oxford, New York.

Gardner, W.J. (1973) The Dysraphic States. Excerpta Medica, Amsterdam, Oxford, New York.

Gerbie, A.B., Melancon, S.B., Ryan, C. and Nadler, H.L. (1972) Cultivated epithelial-like cells and fibroblasts from amniotic fluid: their relationship to enzymatic and cytologic analysis. American Journal of Obstetrics and Gynecology; 14: 314-320.

Gordon, S. and Cohn, Z.A. (1973) The macrophage. International Review of Cytology; 36: 171-214.

Gosden, C.M. and Brock, D.J.H. (1978 a) Combined use of alpha fetoprotein and amniotic fluid cell morphology in early prenatal diagnosis of fetal abnormalities. Journal of Medical Genetics; 15: 262-270.

Gosden, C.M. and Brock, D.J.H. (1978 b) Amniotic fluid morphology in early antenatal prediction of abortion and low birth weight. British Medical Journal; 2: 1186-1189.

Gosden, C.M., Brock, D.J.H. and Eason, P. (1977) The origin of the rapidly adherent cells found in amniotic fluids from foetuses with neural tube defects. Clinical Genetics; 12: 193-201.

Gosden, C.M., Papp, Z. and Ross, A. (1979) Neural cell morphology in amniotic fluid from fetuses with neural tube defects. In: Murken, J.D., Strengel-Rutkowski, S. and Schivinger, E. Eds. Prenatal Diagnosis. Proceedings of the 3rd European Conference on Prenatal Diagnosis of Genetic Disorders. pp. 111-117. Ferdinand Enke Publishers, Stuttgart.

Gosden, C.H., Ross, A. and Eason, P.J. (1981) Amniotic fluid cell cytology and cytogenetics. In: Sandler, M. Ed. Amniotic Fluid and its Clinical Significance. pp. 37-112. Marcel Deken, Inc. New York and Basel.

Green, H. and Thomas, J. (1978) Pattern formation by cultured human epidermal cells: development of curved ridges resembling dermatoglyphs. Science; 200: 1385-1388.

Hamilton, W.J., Boyd, J.D. and Mossman, H.W. (1972) Development of blood. In: Human Embryology. pp. 169-173. W. Heffer and Sons Limited, Cambridge.

Hardisty, R.M. and Weatherall, D.J. (1974) Haemopoiesis during fetal development. In: Blood and its Disorders. pp. 4-5. Blackwell Scientific Publications, Oxford, Edinburgh.

Hasholt, L. (1976) Behaviour of cell cultures from human amniotic fluid. Journal of Medical Genetics; 13: 34-37.

Hawrot, E. (1980) Cultured sympathetic neurons: effects of cell derived and synthetic substrata on survival and development. Developmental Biology; 74: 136-151.

Hay, E.D. (1980) Fibronectin and the extracellular matrix. Current Topics in Developmental Biology; 14: 367-368.

Hayhoe, F.G. and Quaglino, D. (1980) Esterases. In: Haematological Cytochemistry. pp. 146-171. Churchill Livingstone, Edinburgh, London, New York.

Hecht, F., Peakman, D.C., Kaiser-McCaw, B. and Robinson, A. (1981) Amniocyte clones for prenatal cytogenetics. American Journal of Medical Genetics; 10: 51-54.

Henkels-Dully, M.J. and Niermeijer, M.F. (1976) Variation in lysosomal enzyme activity during growth in culture of human fibroblasts and amniotic fluid cells. Experimental Cell Research; 97: 304-312.

Hoehn, H., Bryant, E.M., Karp, L.E. and Martin, G.M. (1974) Cultivated cells from diagnostic amniocentesis in second trimester pregnancies. I. Clonal morphology and growth potential. Pediatric Research; 8: 746-757.

Hoehn, H., Bryant, E.M., Fantel, A.G. and Martin, G.M. (1975) Cultivated cells from diagnostic amniocentesis in second trimester pregnancies. III. The fetal urine as a potential source of clonable cells. Humangenetik; 29: 285-290.

Hoyes, A.D. (1968) Ultrastructure of the cells of the amniotic fluid. Journal of Obstetrics and Gynecology of the British Commonwealth; 75: 164-171.

Huisjes, H.J. (1970) Origin of the cells in liquor amnii. American Journal of Obstetrics and Gynecology; 106: 1222-1228.

Huisjes, H.J. (1973) Cytology of the amniotic fluid and its clinical applications. In: Fairweather, D.V.I. and Eskes, T.K.A.B. Eds. Amniotic Fluid - Research and Clinical application. pp. 95-132. Excerpta Medica, Amsterdam, Oxford, New York.

Huisjes, H.J. (1978) Cytology of the amniotic fluid and its clinical applications. Idem. pp. 93-129.

Jenkins, E.C., Brown, W.T., Duncan, C.J., Brooks, J., Ben-Yishay, M., Giordano, F.M. and Nitowsky, H.M. (1981) Feasibility of fragile X chromosome prenatal diagnosis demonstrated. Lancet; II: 1292.

Jessen, K.R. and Mirsky, R. (1980) Glial cells in the enteric nervous system contain glial fibrillary acidic protein. Nature; 286: 736-737.

Johnson, P.M. and Faulk, W.P. (1978) Immunological studies of human placentae: identification and distribution of proteins in immature chorionic villi. Immunology; 34: 1027-1035.

Kaplow, L.S. (1975) Cytochemical heterogeneity of human circulating monocytes. Acta Cytologica; 19: 358-365.

Kennedy, P. (1980) Personal communication.

Ladermacher, D.S., Vermeulen, R.C.W., Harten, J.J. and Arts, N.F. (1981) Circumvallate placenta and congenital malformation. Lancet; I: 732.

Langman, J. (1981) Central nervous system. In: Medical Embryology. pp. 320-356. Williams and Wilkins, Baltimore, London.

Laundon, C.H., Priest, J.H. and Priest, R.E. (1981) The characterization of hCG regulation in cultured human amniotic fluid cells. Prenatal Diagnosis; 1: 269-275.

Laushlati, K. and Ikonen, S. (1980) Placenta as an indicator of fetal postnatal prognosis. Obstetrics and Gynecology Survey; 35: 145-147.

Lazarides, E. (1980) Intermediate filaments as mechanical integrators of cellular space. Nature; 283: 249-256.

Leijh, P.C.J., van den Barselaar, M. Th., van Zwet, T.L., Dubbeldeman-Rempt, I. and van Furth, R. (1979) Kinetics of Staphylococcus aureus and Escherichia coli by human granulocytes. Immunology; 37: 453-465.

Lim, R. (1980) Glia maturation factor. Current Topics in Developmental Biology; 16: 305-322.

Lim, R., Turriff, D.E. and Troy, S.S. (1976) Response of glioblasts to a morphological transforming factor: cinematographic and chemical correlations. Brain Research; 113: 165-170.

Littlefield, J.W. (1971) Problems in the use of cultured amniotic fluid cells for biochemical diagnosis. Birth Defects: Original Article Series; 7: 15-17.

Lubinska, L. (1973) In: Locomotion of Tissue Cells. Ciba Foundation Symposium 14 (New Series). pp. 228. Elsevier Scientific Publishing Company, Amsterdam.

Lueck, J. and Aladjem, S. (1980) Time-lapse study of normal human trophoblast in vitro. Americak Journal of Obstetrics and Gynecology; 138: 288-292.

Lumsden, C.E. and Pomerat, C.M. (1951) Normal oligodendrocytes in tissue culture. Experimental Cell Research; 2: 103-114.

Marín- Padilla, M. (1970) Morphogenesis of anencephaly and related malformations. Current Topics in Pathology; 51: 145-173.

Martin, A.D. (1980) Characteristics of amniotic fluid cells in vitro and attempts to improve culture techniques. Clinical Obstetrics and Gynaecology; 7: 143-173.

Medina-Gómez, P. (1979) The phagocytic properties and morphology of amniotic fluid and fetal organ cells. M.Sc. Thesis. University of Edinburgh.

Megaw, J.M., Priest, J.H., Priest, R.E. and Johnson, L.D. (1977) Differentiation in human amniotic fluid cell cultures. Journal of Medical Genetics; 14: 163-167.

Meltzer, M.S., Tucker, R.W. and Breuer, A.C. (1975) Interaction of BCG activated macrophages with neoplastic and nonneoplastic cell lines in vitro: cinemicrographic analysis. Cellular Immunology; 17: 30-42.

Mepham, B.L. (1973) Fluorescence microscopy. In: Cook, H.C. Ed. Histopathology: Selected Topics. pp. 245-292. Baillière-Tindall, London.

Michell, R.H., Pancake, S.J., Noseworthy, J. and Karnovsky, M.L. (1969) Measurement of rates of phagocytosis. The Journal of Cell Biology; 40: 216-224.

Middleton, C.A. (1973) The control of epithelial cell locomotion in tissue culture. In: Locomotion of Tissue Cells. Ciba Foundation Symposium 14 (New Series). pp. 251-270. Elsevier Scientific Publishing Company, Amsterdam.

Mirsky, R., Wendon, L.M.B., Black, P., Stolkin, C. and Bray, D. (1978) Tetanus toxin: a cell surface marker for neurons in culture. Brain Research; 148: 251-259.

Moore, K. and McBride, W.H. (1980) The activation state of macrophage subpopulations from a murine fibrosarcoma. International Journal of Cancer; 26: 609-615.

Morris, H.H.B. and Bennett, M.J. (1974) The classification and origin of amniotic fluid cells. Acta Cytologica; 18: 149-154.

Mota, I. (1980) Activity of immune cells. In: Bier, O.D., Dias da Silva, W. Götze, D. and Mota, I. Eds. Fundamentals of Immunology. pp. 33-57. Springer-Verlag, New York, Heidelberg, Berlin.

Mueller, J., Brun del Re G., Buerski, H., Keller, H.U., Hess, M.W. and Cottier, H. (1975) Nonspecific acid esterase activity: a criterion for differentiation of T and B lymphocytes in mouse lymph nodes. European Journal of Immunology; 5: 270-274.

Mulligan, R.M. and Carnes, C.M. (1979) Neural cells in culture. Annals of Clinical and Laboratory Science; 9: 396-407.

Nadler, H.L. (1972) Prenatal detection of genetic disorders. Advances in Human Genetics; 3: 1-37.

Nairn, R.C. (1969) Immunological tracing: general considerations. In: Fluorescent Protein Tracing. pp. 103-138. E & S Livingstone, Edinburgh, London.

Nathan, C.F., Murray, H.W. and Cohn, Z.A. (1980) The macrophage as an effector cell. New England Journal of Medicine; 303: 622-626.

Nelson, M.M. (1973) Amniotic fluid cell cultures and chromosome studies. In: Emery, A.E.H. Ed. Antenatal Diagnosis of Genetic Disease. pp. 69-81. Churchill Livingstone, Edinburgh, London.

Nelson, M.M. and Emery, A.E.H. (1973) Amniotic fluid cell cultures. Journal of Medical Genetics; 10: 19-22.

Nicol, T. and Bilbey, D.L.J. (1958) Elimination of macrophage cells of the reticulo-endothelial system by way of the bronchial tree. Nature; 182: 192-193.

North, R.J. (1978) The concept of the activated macrophage. Journal of Immunology; 121: 806-809.

Oehmichen, M. (1978) Mononuclear phagocytes in the central nervous system. Neurology Series. Springer-Verlag, Berlin, Heidelberg, New York.

Papp, Z. and Bell, J.E. (1979) Uncultured cells in amniotic fluid from normal and abnormal foetuses. Clinical Genetics; 16: 282-290.

Pasquinucci, C., Meroni, P., Dambrosio, F. and Della Torre, L. (1969) The amniotic fluid. Annali di Ostetricia E Ginecologica; 91: 90-106.

Patrick, A.D. (1978) Biochemical studies on amniotic fluid and its cells. In: Scrimgeour, J.B. Ed. Towards the Prevention of Fetal Malformation. pp. 165-174. Edinburgh University Press.

Pettmann, B., Louis, J.C. and Sensenbrenner, M. (1979) Morphological and biochemical maturation of neurones cultured in the absence of glial cells. Nature; 281: 378-380.

Petri, J., Braendstrup, O. and Werdelin, O. (1979) Macrophage-lymphocyte clusters in the immune response to soluble protein antigen in vitro. VIII. Cinephotomicrographic Studies. Scandinavian Journal of Immunology; 10: 493-498.

Pollack, M.S., Maurer, D., Levine, L.S., New, M.I., Pang, S., Duchon, M.A., Owens, R.P., Merkatz, I.R., Nitowsky, H.M., Sachs, G. and Dupont, B. (1979) Prenatal diagnosis of congenital adrenal hyperplasia (21 hydroxylase deficiency) by HLA typing. Lancet; I: 1107-1108.

Pollack, M.S., Ochs, H.D. and Dupont, B. (1980) HLA typing of cultured amniotic cells for the prenatal diagnosis of complement C_4 deficiency. Clinical Genetics; 18: 197-200.

Pomerat, C.M. (1952) Dynamic neurogliology. Texas Reports on Biology and Medicine; 10: 885-913.

Priest, R.E., Priest, J.H., Moinuddin, J.F. and Keyser, A.J. (1977 a) Differentiation in human amniotic fluid cell cultures: I Collagen production. Journal of Medical Genetics; 14: 157-162.

Priest, J.H., Priest, R.E. and Sgoutas, D.S. (1977 b) Production of hormone by cells cultured from human amniotic fluid. American Journal of Human Genetics; 29: 88 A.

Priest, R.E., Marimuthu, K.M. and Priest, J.H. (1978) Origin of cells in human amniotic fluid cultures. Laboratory Investigation; 39; 106-109.

Priest, R.E., Priest, J.H., Moinuddin, J.F. and Sgoutas, D.S. (1979) Differentiation in human amniotic fluid cell cultures; chorionic gonadotrophin production. In Vitro; 15: 142-147.

Priest, R.E., Priest, J.H., Laundon, C.H. and Snider, P.W. (1980) Multinucleate cells in cultures of human amniotic fluid form by fusion. Laboratory Investigation; 43: 140-144.

Rabinovitch, M. and De Stefano, M.J. (1973) Particle recognition by cultivated macrophages. Journal of Immunology; 110: 695-701.

Raff, M.C., Fields, K.L., Sen-itiroh, H., Mirsky, R., Pruss, R. and Winter, J. (1979) Cell-type-specific markers for distinguishing and studying neurons and the major classes of glial cells in culture. Brain Research; 174: 283-308.

Raju, T., Bignami, A. and Dahl, D. (1981) In vivo and in vitro differentiation of neurons and astrocytes in the rat embryo: immunofluorescent study with neurofilament and glial filament antisera. Developmental Biology; 85: 344-357.

Rakic, P. (1975) Cell migration and neuronal ectopias in the brain. Birth Defects: Original Article Series; XI: 95-129.

Report of the United Kingdom Collaborative Study on Alpha Feto-protein in Relation to Neural Tube Defects (1977) Lancet; I: 1323-1333.

Roessmann, U., Velasco, M.E., Sindley, S.D. and Gambetti, P. (1980) Glial fibrillary acidic protein (GFAP) in ependymal cells during development. An immunocytochemical study. Brain Research; 200: 13-21.

Russo, R.G., Liotta, L.A., Thorgeirsson, U., Brundage, R. and Schiffmann, E. (1981) Polymorphonuclear leukocyte migration through human amnion membrane. Journal of Cell Biology; 91: 459-467.

Rutishauser, U., Hoffman, S. and Edelman, G.M. (1982) Binding properties of a cell adhesion molecule from neural tissue. Proceedings of the National Academy of Sciences of the United States of America; 79: 685-689.

Sarkar, S., Cheng Chang, H., Porreco, R.P. and Jones, O.W. (1980) Neural origin of cells in amniotic fluid. American Journal of Obstetrics and Gynecology; 136: 67-72.

Schachner, M., Schoonmaker, G., Hynes, R.O. (1978) Cellular and subcellular localization of LETS protein in the nervous system. Brain Research; 158: 149-158.

Sensenbrenner, M., Maderspach, K., Latzkovits, L. and Jaros, G.G. (1978) Neuronal cells from chick embryo cerebral hemispheres cultivated on poly-lysine coated surfaces. Developmental Neurosciences; I: 90-101.

Seppälä, M. (1977) Immunologic detection of alpha fetoprotein as a marker of fetal pathology. Clinical Obstetrics and Gynecology; 20: 737-757.

Siegel, S. (1956). The Mann-Whitney U Test. In: Non Parametric Statistics for the Behavioral Sciences. pp. 116-127. McGraw-Hill Book Company Inc., New York, St. Louis, San Francisco, London, Mexico, Sydney, Toronto.

Sotelo, J., Gibbs, C.J. Jr., Gadjusek, D.D., Hock Toh, G. and Wurth, M. (1980) Method for preparing cultures of central neurons: cytochemical and immunochemical studies. Proceedings of the National Academy of Sciences of the United States of America; 77: 653-657.

Steele, M.W. and Breg, W.R. (1966) Chromosome analysis of human amniotic fluid cells. Lancet; I: 383-385.

Sternberger, L.A. (1974) Immunofluorescence. In: Osler, A. and Weiss, L. Eds. Immunocytochemistry. Foundation of Immunology Series. pp. 18-55. Prentice Hall, New Jersey.

Stirrat, G.H., Turnbull, A.C., Bennett, H.J., Bobrow, M., Lindenbaum, R.H., Wald, N.J. and Cuckle, H.S. (1979) Clinical dilemmas arising from the antenatal diagnosis of neural tube defects. British Journal of Obstetrics and Gynaecology; 86: 161-166.

Sueoka, N., Chikaraishi, S., Deeb, S., Hsieh, P., Imada, M. and Tomozawa, Y. (1981) Cell type conversion of a rat neural stem cell line in vitro and complexity of gene expression in rat brain. In: Problems in General Genetics. Proceedings of the XIV International Congress of Genetics. II. pp. 144-159. M.I.R. Publishers, Moscow.

Sutherland, G.R., Brock, D.J.H. and Scrimgeour, J.B. (1973) Amniotic fluid macrophages and anencephaly. Lancet; II: 1098-1099.

Sutherland, G.R., Bauld, R. and Bain, A.D. (1974) Observations on human amniotic fluid cell strains in serial culture. Journal of Medical Genetics; 11: 190-195.

Sutherland, G.R., Brock, D.J.H. and Scrimgeour, J.B. (1975) Amniotic fluid macrophages and the antenatal diagnosis of anencephaly and spina bifida. Journal of Medical Genetics; 12: 135-137.

Ten Berge, B.S. (1965) The placenta in anencephaly. Gynaecologia; 159: 359-364.

Trapp, B.D., Honegger, P., Richelson, E. and Webster, H. De F. (1979) Morphological differentiation of mechanically dissociated fetal rat brain in aggregating cell cultures. Brain Research; 160: 117-130.

Tushinsky, R.J., Oliver, I.T., Guilbert, L.J., Tynan, P.W., Warner, J.R. and Stanley, E.R. (1982) Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. Cell; 28: 71-81.

Tyden, O., Bergström, S. and Nilsson, B.A. (1981) Origin of amniotic fluid cells in mid-trimester pregnancies. British Journal of Obstetrics and Gynaecology; 88: 278-286.

Unanue, E.R. (1980) Cooperation between mononuclear phagocytes and lymphocytes in immunity. New England Journal of Medicine; 303: 977-985.

Unanue, E.R. (1981) The regulatory role of macrophages in antigenic stimulation. Symbiotic relationship between lymphocytes and macrophages. Advances in Immunology; 31: 1-136.

Unkeless, J.C., Fleit, H. and Mellman, I.S. (1981) Structural aspects and heterogeneity of immunoglobulin Fc receptors. Advances in Immunology; 31: 247-270.

Valenti, C. (1965) Analysis of trophoblastic cells in vitro by time-lapse photography. American Journal of Obstetrics and Gynecology; 91: 550-556.

van der Meulen, J.A., McNabb, T.C., Hoeffner-Cavaillon, N., Klein, M. and Dorrington, K. (1980) The Fc gamma-receptor on human placenta plasma membrane. I. Studies on the binding of homologous and heterologous immunoglobulin G. Journal of Immunology; 124: 500-507.

van der Veer, E., Kleijer, W.J., de Josselin de Jong, J.E., Galjaard, H. (1978) Lysosomal enzyme activities in different types of amniotic fluid cells measured by microchemical methods. Human Genetics; 40: 285-292.

van Furth, R., Cohn, Z.A., Hirsch, J.G., Humphrey, J.H., Spector, W.G. and Langevoort, H.L. (1972) The mononuclear phagocyte system; a new classification of macrophages, monocytes and their precursor cells. Bulletin of the World Health Organization; 46: 845-852.

van Furth, R. and van Zwet, T.L. (1973) In: Weir, D.M. Ed. Handbook of Experimental Immunology. pp. 36.1-36.24. Blackwell Scientific Publications, Edinburgh.

Virtanen, I., Lehto, V.P., Lehtonen, E., Vartio, T., Stenman, S., Kurki, P., Wager, O., Small, J.V., Dahl, D. and Badley, R.A. (1981 a) Expression of intermediate filaments in cultured cells. Journal of Cell Science; 50: 45-63.

Virtanen, I., von Koskull, H., Lehto, V.P., Vartio, T. and Aula, P. (1981 b) Cultured human amniotic fluid cells characterised with antibodies against intermediate filaments in indirect immunofluorescence microscopy. Journal of Clinical Investigation; 68: 1348-1355.

von Koskull, H., Virtanen, I., Lehto, V.P., Vartio, T., Dahl, D. and Aula, P. (1981) Glial and neuronal cells in amniotic fluid of anencephalic pregnancies. Prenatal Diagnosis; 1: 259-267.

Voss, R., Kohn, G., Saham, M., Benzur, Z., Arnon, J., Ornoy, A., Yaffe, H., Golbus, M. and Auerbach, A.D. (1981) Prenatal diagnosis of Fanconi anemia. Clinical Genetics; 20: 185-190.

Wald, N.J., Cuckle, H.S., Barlow, R.D., Smith, A.D., Stirrat, G.M., Turnbull, A.C., Bobrow, M., Brock, D.J.H. and Stein, S.M. (1980) Early antenatal diagnosis of exomphalos. Lancet; I: 1368-1369.

Weir, D.M. (1977) Immunology. An outline for Students of Medicine and Biology. pp. 8-20 and 59-87. Churchill Livingstone, Edinburgh and London.

Wendon, L.M.B. (1979) Tetanus toxin: its cellular binding, specificity and mechanism of presynaptic interference. Ph.D. Thesis. University College, London.

Wessels, N.K. (1973) Locomotion of tissue cells. Ciba Foundation Symposium 14 (New Series). p. 225. Elsevier Scientific Publishing Company, Amsterdam.

Wintrobe, M.M., Lee, G.R., Boggs, D.R., Bithell, T.C., Athens, J.W. and Foerster, J. (1974) Blood formation in the embryo and the fetus. In: Clinical Hematology. pp. 53-55. Lea and Febiger, Philadelphia.

Wood, G., Reynard, J., Krishnan, E. and Racela, L. (1978 a) Immunobiology of the human placenta. I. Ig Fc receptors in trophoblastic villi. Cellular Immunology; 35: 191-204.

Wood, G., Reynard, J., Krishnan, E. and Racela, L. (1978 b) Immunobiology of the human placenta. II. Localization of macrophages, in vivo bound Ig G and C 3. Cellular Immunology; 35: 205-216.

Yavin, Z. and Yavin, E. (1980) Survival and maturation of cerebral neurons in poly-L-lysine surfaces in absence of serum. Developmental Biology; 75: 454-459.